EFFECTS OF LAMIVUDINE ON BIOCHEMICAL INDICES IN RATS.

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ABSTRACT

Lamivudine (L(-)2[1],3[1]–dideoxy–3-thiacytidine) is an antiretroviral drug which exhibits tissue toxicity leading to peripheral neuropathy and parkinsonism. The exact mechanism of cytotoxicity and effects on target tissues are not well understood. This study was designed to elucidate the effects of lamivudine on biochemical indices in the liver, kidney and brain of rats.

Female Wistar rats (180-200g) were randomly assigned into 5 groups of 6 rats each treated orally for 45 days with normal saline (Control), 4 mg/kg, 20 mg/kg, 100 mg/kg and 500 mg/kg lamivudine respectively. Rats were sacrificed after 12 hours fast and blood (6 mL) collected. Serum obtained was used for biochemical analysis. Serum Alanine and Aspartate Aminotransferases (ALT and AST), Quinine Oxidase (QO), γ–GlutamylTransferase(GGT), urinary trehalase activities as well as urinary creatinine and protein were determined by spectrophotometric techniques while urinary magnesium (Mg^{2+}) level was determined by atomic absorption spectrophotometry. In the liver, kidney and brain, the activities of Superoxide Dismutase (SOD), Glutathione-S-Transferase (GST) and levels of malondialdehyde (MDA, index of lipid peroxidation) were determined. Histology of the liver and kidney was assessed using hematoxylin and eosin stain. Data were analysed using ANOVA at p=0.05.

Lamivudine (500 mg/kg) produced significant increases in the activities of serum ALT, AST, GGT and QO (33.2±3.9, 56.4±7.2, 16.3±1.8 IU/L and 10.1±1.7 Baier's Unit (BU)) relative to controls (21.3±1.5, 42.6±1.9, 11.1±0.7 IU/L and 4.9±1.0 BU) respectively. The drug at 20, 100 and 500 mg/kg increased hepatic GGT activities by 3.1, 4.0 and 5.2 folds while hepatic GST increased by 1.7, 1.8 and 2.0 folds relative to controls respectively. Renal GST activities significantly increased in lamivudine (100 and 500 mg/kg) treated rats (3.15±0.12, 3.57±0.23x 10^{-2} U/mg protein) relative to control (1.74±0.23x 10^{-2} U/mg protein). The level of hepatic MDA in control was 0.06±0.01 nmol/mg protein while lamivudine at 100 and 500 mg/kg significantly increased MDA levels (0.17±0.03, 0.22±0.04 respectively). Also, lamivudine at 100 and 500 mg/kg significantly increased renal MDA levels by 125% and 189% respectively when compared with the control. The activity of hepatic SOD in control was 9.9±0.7 U/mg protein. Treatment with lamivudine (20, 100 and 500 mg/kg) significantly increased SOD activities (14.7±1.5, 18.4±1.2, 16.5±1.3 U/mg protein). Similar trend was observed for renal SOD

activities of rats treated with lamivudine. Administration of lamivudine at 100 mg/kg significantly increased urinary trehalase activity (324.1±15.3 vs 157.8±18.6 U/mg per ml), urinary Mg^{2+} concentration (8.6±0.4 vs 4.8±0.5x10^{-3}/mg/ml) and urinary protein (2.9±0.29 mg/ mlvs 1.4±0.14mg/ml). However, there were no significant differences in the values of brain SOD, MDA, and urinary creatinine in lamivudine-treated animals relative to controls. Histological sections of rats treated with lamivudine (100 and 500 mg/kg) showed visible lesions in the liver (hydropic degeneration) and kidney (cortical congestions).

Repeated administration of lamivudine altered biochemical indices accompanied by visible histologic effects in the liver and kidney of the rats. The biochemical alterations appear to be mediated by oxidative stress.

Keywords: Biochemical Indices, Cytotoxicity, Lamivudine, Oxidative stress.
Word count: 484

CERTIFICATION

I certify that this work was carried out by **Mr. LamidiWaheedBabatundeOLANIYAN** in the Department of Biochemistry, University of Ibadan.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

1.0INTRODUCTION

The use of nitrogen mustards and of folic acid inhibitors in cancer treatments in the 1940s probably heralded the era of chemotherapy(Farber *et al*., 1948; Goodman *et al*., 1946). Chemotherapy originally associated with cancer treatments using drugs, has been extended to mean treatment of all forms of ailments with synthetic chemicals or drugs. Antineoplastic therapy and cytotoxic therapy are the contemporary coinage for cancer treatment drugs. Synthetic remedies or chemotherapeutic agents are available, offering permanent cure to almost all diseases and carrying their toxicities along thereby limiting their benefits.

Drug toxicity, the systemic effects of a drug that are related to the overall level of the medication in the bloodstream, mayoccur with over-dosage of a medication, accumulation of the drug in the body overtime or the inability of the patients body to eliminate the drug. Toxicity and adverse reactions are twin properties of chemotherapeutic agents which must be studied and understood properly for safe and effective healthcare delivery.Quinine and mefloquine are well known two of the mainstay for treating severe malaria, a disease known for its notoriety in the Tropics. While quinine is known for its hypoglycaemic effect, mefloquine causes dose - related serious neuropsychiatric toxicity (Alkadi, 2007). Troglitazone (TRO), a thiazolidine compound was a novel anti-diabetic agent originally approved to treat patient with adult onset non-insulin dependent diabetes (Lehman *et al.*,1995). TRO was known to sensitize target cells to insulin thereby improving the metabolic conditions associated with adult onset diabetes. The drug had to be withdrawn from the market (Bac and Song, 2002) for causing severe hepatic problems with elevated severe transaminase activities (Whatkins and Whitcomb,1998).

At least three well-known tragedies have occurred as a result of imperfect knowledge of the toxic potential of the drugs being administered. The first is the sulphanilamide elixir which occurred in 1937 in the United States. A sulphanilamide elixir that employed 72% diethylene glycol as a solvent was made available for the treatment of systemic infections. Within 3 days of consuming this elixir more than 100 persons died of nephrotoxicity induced by the solvent. A second example concerned the antibiotic, chloramphenicol. Before its toxicity was fully appreciated, the drug was used indiscriminately for virtually all types of bacterial infections. The drugs produced

the green baby syndrome in infants especially premature infants characterized by vasomotor collapse, cyanosis, among others and finally death. (Weiss *et al.*,1960). The third tragedy was the thalidomide disaster. The drug was used as an effective hypnotic drug in the late 1950s and 1960. During the period, the number of infants born with deformed limbs (phocomelia) increased(Taussing, 1962) when the drug was taken by their mothers during pregnancy.

Drug – induced toxicity in a biologic model is caused by either the parent drugs or their metabolites. The toxicity of acetaminophen for example, is believed to be associated with cytochrome P-450 -mediated generation of a hepatotoxic metaboliteN-acetyl–p-benzoquinoneimine(Dahlin*etal.*,1984). Metabolic studies conducted on the felbamate, an anticonvulsant drug (Macobe *etal.,* 1993) has led to the design of a more potent less toxic fluorofelbamate (Rocklein *et al.*, 2007).

In the contemporary world of chemotherapy, antiretroviral drugs have succeeded in extending the lives of HIV – infected patients. Unfortunately the drugs benefits are beset with drug – induced toxicities leading to, in some cases the withdrawal of some drugs from the circulation.Hereinliesthe imperativeness of sound knowledge of drug toxicity in the delivery of safe and effective health care.

Human immunodeficiency virus infection is a disease caused by human immunodeficiency viruses 1 and 2 (HIV-1&2) with presently no known cure. The disease is currently being managed by cocktails of life extending drugs which reduce body burden of viral load and subsequently preventprogression to full blown acquired immunodeficiency syndrome (AIDS). The cocktail or HAART (highly active antiretroviral therapy) (Larder, 2001) is the standard of care since 1996 (Palella, 1998) containing at least three antiretrovirals(ARV) (Zapor *et al.,* 2004) which may be drawn from the available pharmacological classes of ARV (Weller and Williams, 2001). However, the benefits of HAART are beset with tremendous pill burdens, side effects, toxicities and complications from drug interactions (Yeni *et al.,* 2002). Strict adherence to the cocktail is imperative to limit resistance to the drugs.

Nucleoside analogue reverse transcriptase inhibitors (NRTI) belong to the pharmacological classes of ARV (Weller and Williams, 2001). Their therapeutic action on HIV infection is to prevent formation of the viral DNA by inhibiting HIV reverse transcriptase. Nucleoside

analogues act as alternative substrates for DNA polymerases. They lack a hydroxyl group in the 3^1 position required for the addition of the next nucleotide on to the primer, their incorporation causes termination of the growing DNA strand. However, NRTIs have the potential to cause serious cellular toxicities by interacting in a similar manner with human DNA polymerase (Brinkman *et al.*, 1998). Many side effects of NRTI are believed to stem from their effect on polymerase gamma, the enzyme responsible for mitochondrial DNA replication, and the resulting mitochondrial dysfunction (Brinkman *et al.*, 1999; Brinkman *et al.*, 1998). Clinical effects attributed to mitochondrial dysfunction include lactic acidosis and neuropathy (Marfatia and Makradi, 2005; Lewis and Dalakas, 1995).

HIV/AIDS drugs have been withdrawn from the market due to serious toxicity. Treatment with Fialuridine, a drug in the class of NRTI had been reportedly terminated in patients because of the drug´smitochondrial damage exhibited by severe clinical multisystemic toxicity (Makenzie *et al.*, 1995; Swartz, 1995).

Lamivudine, (2R-cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H-pyrimidine-2-one)(Harris *et al*., 1997) or unnatural enantiomer L(-) -2^1, 3^1- dideoxy-3-thiacytidine (3TC) is a NRTI pro-drug that requires metabolic activation to 5^1–triphosphorylated form (Stein and More, 2001) for antiviral activity.The mean 50% inhibitory concentration (IC_{50}) of 3TC against various strains of HIV- 1 and HIV-2 in $CD4^+$ lymphocyte cell lines was reported to range from 4nM to 0.67µM (Coates *et al.,1992*). Aside its anti-HIV activity, 3TC is also known to block hepatitis B virus replication(Doong *etal.*,1991). Lamivudinewas the first to provide *in vivo* evidence of T – cell tolerance induction by high viral load and viral antigen expression in humans(Boni *et al.*1998). By reducing viral load and viral antigen expression with lamivudine, hyporesponsiveness of T – cells to viral protein have been shown to be reduced. Lamivudine is reputed to have a safety record at least more than most of the drugs in its pharmacological class.Its reported adverse effects in the literatures include headache, insomnia, nausea, vomiting, diarrhoea, abdominal pain, eczema, hepatitis, peripheral neuropathy, red cell aplasia and pancreatitis, most of which have been reported to be moderate (European Medicines Agency, 2010).

Although lamivudine has been described as a weak inhibitor of polymerase gamma (Chen *et al.*, 1991), its adverse effects such as peripheral polyneuropathy and pancreatitis thought to be

related to mitochondrial dysfunction follow polymerase gamma inhibition (Kamps and Hoffmann 2007; Fodale *etal.*, 2005). The drug is hardly used in monotherapy presumably because of rapid development of resistance to it by the virus, however its combination with other drugs for effective therapy may mean combining toxicities with dire consequencies. The side effects of these ARVs including 3TC have been associated with damage to the mitochondria, the major cellular source of reactive oxygen species, which their uncontrolled production may lead to oxidative stress and finally death. Enough laboratory study appeared not have been done on 3TC;much of the information available about the drug are anecdotal, that is, based on clinical experience. Intensive laboratory study is *sine qua non* for the purposes of management of adverse drug effects.

1.1 AIMS AND OBJECTIVES OF THE PROJECT

Therefore, the goal of this project was to investigate biochemical and histological changes associated with the drug toxicities in the rat tissues. Specifically, the project was aimed at studying the mechanism of the drug toxicity in rat following long and short – term oral administrations of the drug at low and high dose levels.The results of the investigations are expected to provide a lead into the management of the lamivudine – related adverse effects.

CHAPTER TWO

2.0LITERATURE REVIEW

2.1 ANTIRETROVIRAL THERAPY : AN OVERVIEW

It was not until mid 80s that hope rose for HIV – infected patients with the introduction of anti – HIV drugs, before then the patients were left to die in hospices (Hoffmann and Mulcahy, 2007). Even then, the cure for HIV/AIDS has remained elusive till date. The antiretroviral drugs merely increases the life expectancy of the patient by reducing the body burden of viral load thereby preventing the disease progression to full blown AIDS and its associated complications (Palella *et al.,* 1998).

Zidovudine or AZT, a thymidine analogue and prototype drug in the class of nucleoside analogue reverse transcriptase inhibitor (NRTI) was the first antiretroviral drug licensed by U.S. Food and Drug Administrationin 1987. Other antiretroviral drugsin the group include Zalcitabine (ddC), Stavudine (d4T) and Lamivudine(3TC). ddC was withdrawn from the market in 2006 on account of toxicity and weak antiviral activity (Hoffmann and Mulcahy, 2007). Tenofovir is a nucleotide reverse transcriptase inhibitor drug, though possesses phosphate in its structure, it requires phosphorylation to its diphosphate to exert antiviral activity (Warnke *et al.* ,2006).They are generally not metabolized by hepatic cytochrome P – 450. Their metabolism depends on intracellular enzymes such as nucleoside kinases, 5´ - nucleotidases, purine and pyrimidine nucleoside monophosphate kinases. NRTIs lack OH group at 3^1 position necessary for the formation of the $5^1 \rightarrow 3^1$ phosphodiester bond essential for DNA elongation. Thus NRTIs compete with natural substrates for the reverse transcriptase binding site and incorporation into viral DNA to act as chain terminator in the synthesis of proviral DNA (Mitsuya and Broder, 1986). NRTIs are pro-drugs which require metabolic activation in a stepwise manner to their 5^1 – triphosphates by cellular kinases to exert their antiviral activity (Gao *et al.*, 1994).

Nonnucleoside reverse transcriptase inhibitors (NNRTI) is another class of antiretroviral drugs exemplified by nevirapine (NVP), efavirenz (EFZ) and delavirdine (DLV) (Hoffmann and Mulcahy, 2007). The drugs in this class have no structural similarity. They however share the same mechanism of action. They bound noncompetitively to the reverse transcriptase at the allosteric site thereby causing conformational changes and disrupting the catalytic site of the

enzyme (Spence *et al.*, 1995). NNRTIs are metabolized by hepatic cytochrome P450s. NVP and EFV are inducers of hepatic cytochrome (CYP) 3A4 whereas DLV inhibits the enzyme (von Moltke *et al.*,2001). EFV is also known to inhibit CYP 2C9 and 2CI9 though to a lesser extent. Drug interactions are therefore important consideration when using NNRTIs.

Protease inhibitor drugs (PIs) inhibit HIV – 1 protease by binding to its active site. The enzyme, a symmetric dimer is responsible for the cleavage of the large viral precursor polypeptide chains into smaller, functional proteins thus allowing maturation of the HIV virion. The inhibition results in the release of structurally disorganized non – infectious viral particles (Pillay *et al.*,1995). Examples of PIs are Tipranavir (TPV), Amprenavir (APV), Indinavir(IDV), Saquinavir(SQV) and Ritonavir (RTV) (Hoffman and Mulcahy, 2007). PIs are metabolized by CYP 450 primarily CYP 3A4 with some members as inhibitors (e.g. RTV) or as inducers (e.g Lopinavir LPV).

Enfuvirtide (T- 20) is a 36 – amino acid peptide anti HIV -1 drug (Warnke *etal.,* 2006). The drug bounds to a specific region of virus gp 41 and blocks the formation of the 6 –helix bundle necessary for the fusion of the virus and host cell members and thus viral entry. The binding of the virus membrane glycoprotein gp 120 to CD4 and chemokine receptors causes a conformational change in the structure of gp 41 forming a 6 – helix bundle structure (Warnke *etal.*, 2006). T -20 is the only drug to date in the class of fusion inhibitor. The drug is not orally bioavailable and is therefore administered subcutaneously by the injection. Fusion inhibitors provide one mechanism of entry inhibition.

Other mechanismsof entry inhibition are the attachment inhibition brought about by blocking the binding sites on either CD4 receptors or gp120 and the use of co-receptor antagonists (Steal, 2005).

Naphtyridine carboxamide is the active ingredient in Raltegravir, an integrase inhibitor. Integrase, a viral enzyme coded by pol gene, is one of the three enzymesalong with reverse transcriptase and protease, required for viral replication. Raltegravir has potency against R.5 and X 4 (M – tropic and T – tropic respectively) viruses and also against HIV – 2. In monotherapy, the drug has been reported to reduce viral load in ten days (Markowtiz *et al.*, 2006). Elvitegravir drug is an integrase inhibitor (Sato, 2006).

Maturation inhibitors inhibit HIV replication in the late phase of the replication cycle, that is, the budding or maturation of new virions (Li, 2003). Drug in this class is Bevirimat, a derivative of betulinic acid isolated as triterpene carbonic acid from birch bark (Hoffmann and Mulcahy, 2007).

In addition to the conventional ART, immunomodulatory treatment strategies have been adopted. Interleukine -2 (IL -2) has been used for treating HIV infection (Wood *et al.*, 1993). Treatment with cycles of the cytokine (IL -2) has resulted in substantial increase in CD4 counts and may also improve immune responses to HIV infection (Weller and Williams, 2001) but has little effect on plasma viral load levels (Vento *et al.*, 2006; Kovacs *et al*., 2005; Kovacs *etal.*, 1996).

Drug resistance by HIV and the consequent loss of antiviral activity is often exhibited in monotherapy. Subsequently, combination therapy has become the standard of care in particular for patients with moderate to advanced disease (Gulick *et al*., 1997). Combination therapy or HAART is a cocktail of at least three antiretrovirals (ARVs).

2.2 ADVERSE EFFECTS OF ANTIRETROVIRAL DRUGS

Drugs toxicity issues have dampened the enthusiasm that followed antiretroviral therapy (Lenert *et al.*, 2002;Brinkman and Kakuda, 2000). Nucleoside and nucleotide analogue reverse transcriptase inhibitors (NRTI) play a central role in the treatment of HIV. Toxicities attributed to them especially after prolonged use are lactic acidosis, peripheral neuropathy, myopathy , pancreatitis and lipoatrophy among others (Khilinani *et al.*, 2003; Powderly,2002; Brinkman and Kakuda, 2000). Cellular activation is one critical factor associated with NRTI toxicity. Most of the clinical manifestations of NRTI toxicity resemble mitochondrial diseases. Histologic evidence has demonstrated abnormal mitochondrial DNA (mtDNA) depletion in the affected tissues (Dalakas *et al.*, 2001; Kakuda, 2000). This may be related to the effects on the mitochondrial polymerase gamma, the enzyme responsible for mitochondrial DNA replication. Activated NRTIs (NRTI triphosphates) competitively inhibits mtDNA polymerase gamma (Martin *et al.*, 1994) which in turn decreases the number of respiratory chain proteins, inhibit aerobic respiration, induce oxidative stress, increase mutation in DNA and finally tissue failure (Walker and McComsey, 2007; Lewis *et al.*, 2001).

Adverse effects frequently reported in NNRTI therapy include mild rash (Bardsley – Elliot and Perry, 2000;Scott and Perry, 2000), asymptomatic elevation of liver enzymes (Dieterich *et al.*, 2004) and lipodystrophy (Adkins and Noble, 1998). All protease inhibitors (PIs) to a greater or lesser extent are associated with lipodystrophy and dyslipidemia (Nolan, 2003). Cardiac arrhythmias (Anson, 2005) and sexual dysfunction have also been attributed to PIs (Schrooten, 2001). All PIs inhibit CYP 3A4 system (Hoffmann and Mulcahy, 2007). Adverse reactions presented by T -20, a fusion inhibitor is the so called injection site reactions manifested among others by erythema, induration and ecchymosis (Warnke *et al.*, 2006).

2.3THE DRUG – LAMIVUDINE (3TC)

One of the standard components in the treatment of AIDS that has resulted in dramatic decrease in AIDS-related deaths is lamivudine brand named **Lamivir** or **Epivir**

2.31 NOMENCLATURE

Lamivudine is a laevorotatory pyrimidinone – 1, 3 – oxathiolane derivative with molecular formula $C_8H_{11}N_3O_3S$ (Fig. 1), molecular weight 229.26g/mole (PubMed, 2008). Its IUPAC nomenclature is 4 – amino – pyrimidine – 2 – one (the sulphur atom in place of the 3′ carbon of the ribose ring of 2′ - deoxycytidine) (GlaxoSmithKline, 2006; Soudeyns *et al., 1991*) hence the name 2′,3′ – dideoxy - 3′ – thiacytidine (3TC).

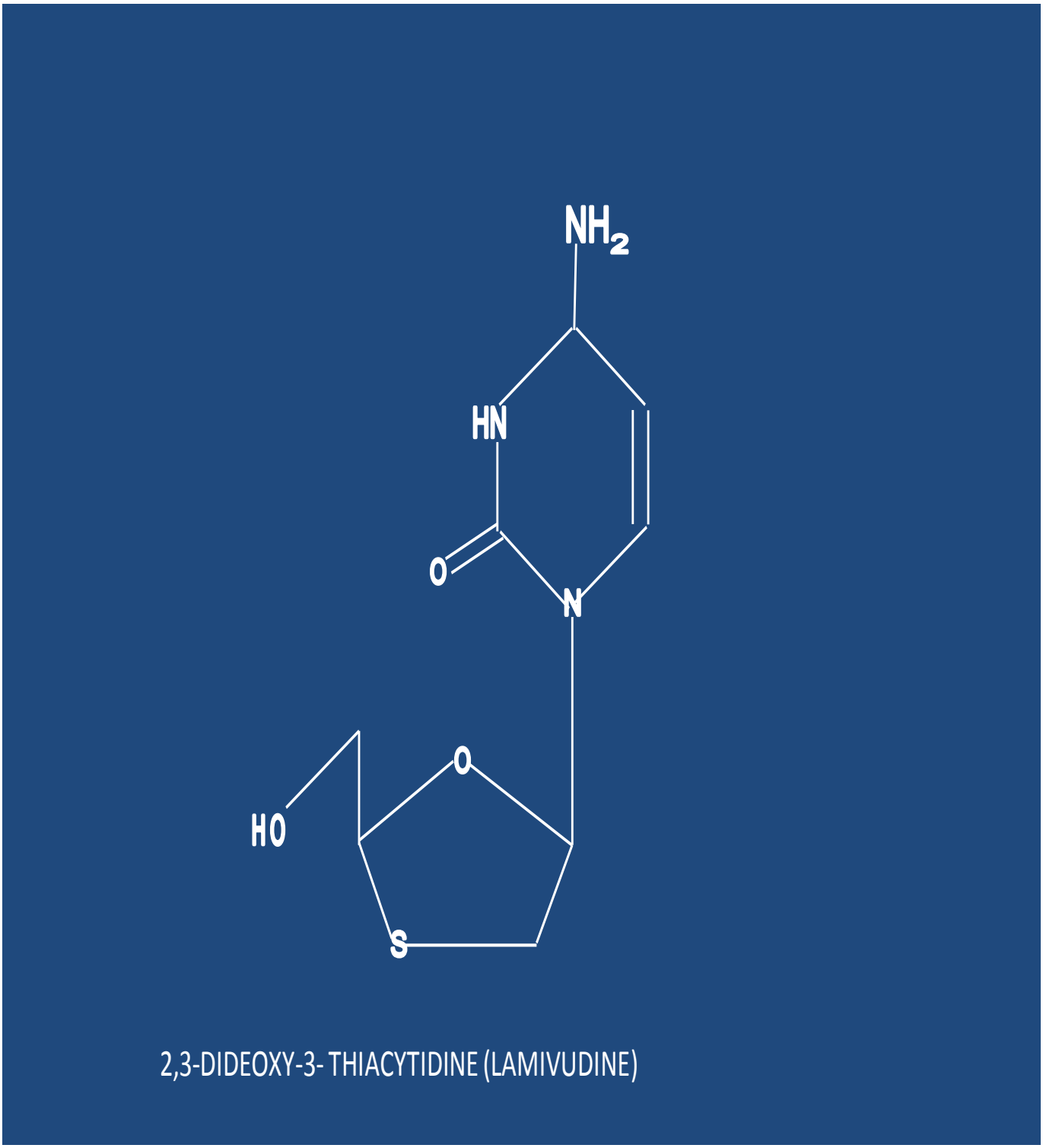


Figure 2.0 Structure of lamivudine

2.32 LAMIVUDINE SOLUBILITY

The World Health Organization (WHO, 2006) has classified lamivudine as soluble in water. The solubility has been reported as 70mg/ml at 20^0C (Martindale, 2010).According to Fernandes *et al.,2006,* the solubility of lamivudine in water at 15^0C was 52.8mg/ml, at 25^0C, 84.9mg/ml and at 35^0C, 149.6mg/ml. Lamivudine is a weak base (pKa , 4.3) protonated at NH_2 group (Koshuba *et al.,1999;* Jozwiakowski *et al., 1996*). Lamivudine melts at 160 – 162^0C (Martindale, 2010).

2.33CLINICAL BENEFITS OF LAMIVUDINE

Lamivudine (3TC) is indicated for the treatment of viral infections such as hepatitis B(Alizadeh *et al.*, 2006; Lee *et al., 2003*) and HIV/AIDS. The drug has potent activity against HIV-1 and HIV-2. It′s mean 50% inhibitory concentration (IC_{50}) against viruses in CD 4+ lymphocyte cell lines ranged from 4µM to 0.67µM (Coates *et al., 1992*).

2.34MECHANISMS OF ANTI – VIRAL ACTIVITY

The cytidine analogue drugmarkedly inhibits hepatitis B virus (HBV) replication both *in vitro* (Doong *et al.*, 1991) and *in vivo* (Dienstag *et al.*,1995).Incorporation of the drug active triphosphate (3TCTP) into growing DNA chains results in premature chain termination thereby inhibiting HBV DNA synthesis. By the same token, lamivudine inhibits HIV reverse transcriptase (RNA – dependent DNA polymerase) (Pluda *et al.*, 1995). The drug requires stepwise intracellular metabolic activation to its 5′ – triphosphate derivative by cytosolic deoxycytidine kinase. The phosphorylation requires cellular rather than viral enzymes, the conversion therefore occurs in both the infected and uninfected cells (Balzarini *et al.*, 2002). Metabolic activation of 3TC is cell – cycle independent, implying the occurrence of the event in actively dividing as well as in resting cells (Balzarini *et al.*, 2002). The inhibition of the viral reverse transcriptase results in viral DNA chain termination. *In vitro*, 3TC acts synergistically with AZT and inhibits AZT-resistant virus (Viner *et al.*,1993).

Prolonged monotherapy with 3TC leads to resistance which is associated with the development of an amino acid substitution valine or isoleucine for methionine(M184V/I) in HIV -1- reverse transcriptase (Schuurman *et al.*,1995). The resistance is thought to involve steric hindrance of reverse transcriptase by the beta- branched amino acids (Sarafianos *et al.*, 1999).

2.35LAMIVUDINE PHARMACOKINETICS

Lamivudine dissolves rapidly once in the stomach and is absorbed in the small intestine by passive diffusion. Food delays but does not alter the extent of absorption when co-administered (European Medicine Agency, 2010). Absolute bioavailability is reported to be 82% in adults and 68% in children (Johnson *et al., 1999;* Perry and Faulds, 1997)with a mean volume of distribution (Vd) of 1.3L/kg indicating considerable distribution into deeper tissues (Leuwen *et al.*,1992). Mean elimination half – life has been reported to be 2.5 hours while the intra –cellular half life of the active 5^1 – triphosphate metabolite is 10.5 - 15.5 hours in HIV infected cell lines (Safrin, 2001). Systemic clearance was 0.33±0.06 L / hr /kg while renal clearance was 0.22±0.061 L / hr /kg.

The brain volume of distribution of 3TC had been reported to be low, less than 0.05ml/100g (Wu *et al., 1998*). The concentration of the drug in the cerebrospinal fluid (CSF) of humans and primates is also low (Mueller *et al.,1998;*Blaney *et al.,1995;*Leeuwen *et al.,1995*). 3TC permeates CSF/brain barriers slowly. In the adults CSF:plasma ratio ranged from 0 to 46% (2 – 4hr dosing) and in children 4 – 8% (2hr dosing). These observations might have led to the proposition that 3TC uses active efflux transport systems to leave central nervous system (Wu *et al., 1998;*Blaney *et al., 1995*). 2′3′ – dideoxyinosine (ddI), another NRTI family of anti – HIV drug, interacts with 3TC at the choroid plexus thereby reducing 3TC accumulation in the brain (Gibbs *et al*. 2003).

Lamivudine undergoes minimal metabolism and renal clearance of the unchanged drug is the predominant mechanism of clearance (Takubo*et al*.,2000). Trimethoprim/Sulphamethoxazole (cotrimoxazole and sulfamethoxazole 160/800) increases 3TCarea under the curve(AUC) but decreases renal clearance when 3TC is administered before and after a 5 – day course of cotrimoxazole (Moore *et al.,* 1996).Studies have demonstrated that the placental barrier is permeable to lamivudine with the materno – fetal ratio of about 1.0 (El Beitune and Duarte, 2006).

2.4METABOLISM OF LAMIVUDINE

Lamivudine enters hepatocytes through active uptake by pyrimidine nucleoside transporters (Torresi and Locarnini, 2000; Mercader *et al.,* 1996). Lamivudine is a pro-drug. In the cytoplasm

of the hepatocytes, it is phosphorylated (activated) to the mono– (3TCMP), di– (3TCDP), and triphosphate (3TCTP) by deoxycytidine kinase, cytidine monophosphate kinase and pyrimidine nucleoside diphosphate kinase respectively. The diphosphate is present in high concentrations inside the hepatocytes and its conversion to triphosphate is thought to be rate limiting step (Kewn *etal.*,1997).

Less than 10% of 3TC is metabolized by the liver, only 5 – 10% of an oral dose of 3TC after 12 hours is metabolized to trans –sulphoxide and excreted in the urine (Johnson *et al.*,1999; Heald *et al.*,1996). The serum level of the sulphoxide has not been precisely determined. It is howeverunlikely that oxidation to sulphoxide is mediated by CYP 450 since no interaction has been reported toexist between the drug and nevirapine, an inducer of CYP 450 (Sabo *et al.*, 2000).

2.41 DRUG INTERACTION

Lamivudine interactionwith some drugs have been reported to elicit certain clinical and biochemical responses in man and experimental animals. For example 3TC competitionfor the phosphorylation process with Cladribine (Chtioui, 2009) and Zalcitabine (GlaxoSmithKline, 2006; Johnson *et al., 1999*) results in inhibition of their activities.Co-administration of the antimalarial Artesunate results in altered glucose tolerance (Olurishe *et al., 2012*). Mechanisms of the interactions have not been elucidated.

Hydroxyurea, a ribonucleotide reductase inhibitor, and methotrexate or fludarabine have been reported to increase 3TC phosphorylation (McGuire *et al.,2003*; Kewn *et al.*,2000) favouring the ratio 3TCTP/dCTP (its natural competitor) thereby potentiating the antiviral activity of 3TC.

Trimethoprim/Sulphamethoxazole has been reported to reduce 3TC excretion ratio in the urine and thus increases blood levels, possibly competing for renal tubular secretion. (Khilinani *et al.*, 2003; Moore *et al.,* 1996). Both clinical and biochemical implications of this have not been precisely determined.

The interaction of 2^1,3^1 – dideoxyinosine (ddI), another NRTI with 3TC at the choroid plexus was reported to reduce 3TC accumulation in the brain (Gibbs *et al., 2003*).

2.42LAMIVUDINE TOXICITIES

Serious toxicities have not been reported of lamivudine relative to other NRTIs, at least at clinical levels (Pluda *et al.*, 1995; Leeuwen*et al.*, 1995;Leeuwen *etal.*,1992) but granulocytopenia , depression and peripheral neuropathy , lactic acodosis as well as pancreatitis have been reported as adverse effects (Lai *et al.*,1998). Parkinson's disease had also been associated with long term drug therapy (Li *et al.*,2007). Children on 3TC therapy had been found to have impaired oral glucose tolerance test, an indication of possible pancreatitis (Ertekin *et al.*, 2005). Significant increase in blood glucose levels was reported among HIV – pregnant women taking triple 3TC – containing ARV drugs (El Beitune *et al.*, 2005).

3TC has been reported to be cytotoxic in human peripheral blood lymphocytes (Coates *et al.,1992;* Sommadossi *et al.,1992*).Pure red cell aplasiaresulting in severe anaemia was reported among lamivudine – treated patients probably confirming the drug's haematotoxicity (John *et al.*, 2008; Weitzel *et al.*,1999;Hester and Peacock , 1998).Umar and colleagues (2008)reported adverse hepatic events in rats orally exposed to 3TC at concentrations higher than the therapeutic dose for HIV infection but the mechanism of hepatotoxicity was not defined.

Morphologic damage to mitochondria had been reported in the hearts of infant *Erythrocebus patas* monkeys perinatally exposed to 3TC – containing NRTIs (Divi *et al.*, 2005).

Foster and co-workers(2004) had suggested that 3TC could be nephrotoxic.

CHAPTER THREE

3.0 MATERIALS AND METHODS

All the reagents used for the experiments were of analytical grade. Reagents for everyday use were supplied by a contractor otherwise they were supplied by either Sigma – Aldrich® or Fluka®. The drug, **Lamivir - 150** brand from **Cipla** was kindly supplied free - of - charge by **FACSEAT-TROPICAL**, a Malawi – based Non - Governmental Organization.

3.1 Drug Sample Preparation.

Each drug contained 150 mg lamivudine (3TC). The drugs were dissolved in known volume of normal saline at the laboratory temperature to give a standard solution. Serial dilutions were thereafter carried out to give the required drug concentrations.

3.2 Experimental Animals

Adult rats, *Wister* strain all females weighing from 150 to 200 g, were used for the animal experiments. They were acclimatized to the laboratory conditions for one week subsisting throughout the experiments on animal chow and potable water *ad libitum*. No sign of ill –health was noticed in the animals before the commencement of the experiments. The animals were appropriately marked for identification and housed in plastic cages individually.

3.3 Experimental Procedures

3.3.1 *In vitro* toxicity study.

Doses of 4, 20 , 100, 500 and 2500 mg 3TC/kg body weight respectively in a total volume of 0.5 ml normal saline was injected into the chorioallantoic sac of 10 days old live embryonated eggs of *Gallus domesticus*, average weight 55 g at 30 eggs per dose level. The control eggs received 0.5 ml normal saline only. The eggs were incubated at 37^{0}C in commercial incubator and candled daily. Both dead and live embryos were autopsied and examined for gross pathological signs after eleven days.

3.3.2 Single oral dose study.

Four dose levels were adopted and spread in geometric progression according to the method of Weil (1952). They were 4, 20, 100, and 500 mg 3TC/kg body weight (b.w.) in single dosing. The start dose (4 mg 3TC/kg) was the daily human therapeutic dose and subsequent doses were in multiples of five (Yemitan and Adeyemi, 2004; Qurestu *et al.*, 1992) . The doses in normal saline solution, respectively in a maximum volume of 2 ml. were orally administered to the adult female rats early in the mornings.

3.3.3 Repeated oral dose study

Similar dose regimens as in the single dosing were administered orally to adult female rats daily for 45 days.

The control groups in both (single and repeated) cases received no drug but 2 ml of normal saline, the medium in which the drug was administered. There were 6 animals per dose level in the studies which were selected randomly. Animals individual weights were determined before and after the experiments. Observation periods were 14 and 45 days respectively. Overnight urine samples were collected before and 24 hours after dosing from the animals in their individual metabolic cages. Records of weight changes were taken. The animals were sacrificed through cervical dislocation respectively on the14th or 45th day of dosing.

3.4 Sample Preparations.

Blood samples from the animals were collected through heart puncture in vacutainers. The clotted blood samples were spun at 3000g for 5 minutes to obtain serum samples.

The livers, kidneys and brains of the animals on repeated doses carefully dissected, were rapidly removed and washed off blood and connective tissues with 1.15% ice cold KCl solution, their

weights thereafter determined. They were stored in ice-cold 0.25 M sucrose solution buffered at pH 7.4 with 40 mM Tris.HCl .

The livers and the kidneys were cut into slices preparatory for homogenization by a motorized homogenizer (10 strokes per sample). Tissue slices of each organ were fixed in Bouin solution for histopathological studies.

3.4.1 Preparation of tissue sub – cellular fraction.

20% (w/v) homogenates of the organs were prepared in ice – cold 0.25 M sucrose solution adjusted to pH 7.4 with 40 mM Tris.HCl buffer. Each homogenate was centrifuged at 8000 x g for 10minutes in a refrigerated ultracentrifuge. (Beckman Ultima – Le 80k). The pellet was taken up in 10 ml of ice – cold 0.25 M sucrose solution and re - centrifuged. It was then dissolved in a total volume of 10 ml of the sucrose solution. This was the mitochondrial fraction. The supernatants were re - centrifuged at 12000 x g for 10 minutes to remove light mitochondria (Kaushal *et al.,* 1999). The products were combined and used as post – mitochondrial supernatant for the biochemical analyses.

3.5 Analytical Methods

3.5.1 Serum ALT activity determination.

Alanine aminotransferase (ALT) or glutamate pyruvate transaminase (GPT) activities were determined using RANDOX laboratories (UK) reagent kits, based on the method described by Reitman and Frankel (1957).

Principle

α- ketoglutarate + L - alanine —ALT —→ L- glutamate + pyruvate .

ALT activities were monitored by measuring the absorbance of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine at 546 nm, against reagent blank. Enzyme activity units (U/L) were read off from the plots of Absorbance versus Enzyme activity unit (Figure 3.1). Data (Table 3.1) were provided by the manufacturer, contained in the manufacturer's instruction manual (AL100) which was supplied along with the reagents.

Reagents

Phosphate buffer 100 mmol/l, pH 7.4

L- alanine 200 mmol/l

α-ketoglutarate 2.0 mmol/l

2, 4-dinitrophenylhydrazine 2.0 mmol/l

Procedure

Wavelength 546 nm

Cuvette 1cm light path

Incubation temperature 37^0C

Contents of the test tubes were as follows:

	Reagent Blank	Test
Sample	-	0.1ml
Buffer	0.5ml	0.5ml
Distilled water	0.1ml	-

The test tubes were shaken and incubated at 37^0C for 30 minutes and thereafter 0.5 ml of 2,4 – dinitrophenylhydrazine was added to each test tube. The contents of the test tubes were agitated again and allowed to stand for 20 minutes at 20^0C after which 5 ml 2.5 M NaOH solution was added to each tube. The absorbance of the sample was read after 5 minutes against the reagent blank.

Table 3.1 Data for serum ALT activity standard curve.

Absorbance (λ = 546 nm)	Enzyme activity (U/L)
0.025	4
0.100	17
0.175	29
0.250	43
0.325	57
0.400	72
0.475	88
0.500	94

Source: RANDOX Laboratory Manual for ALT.

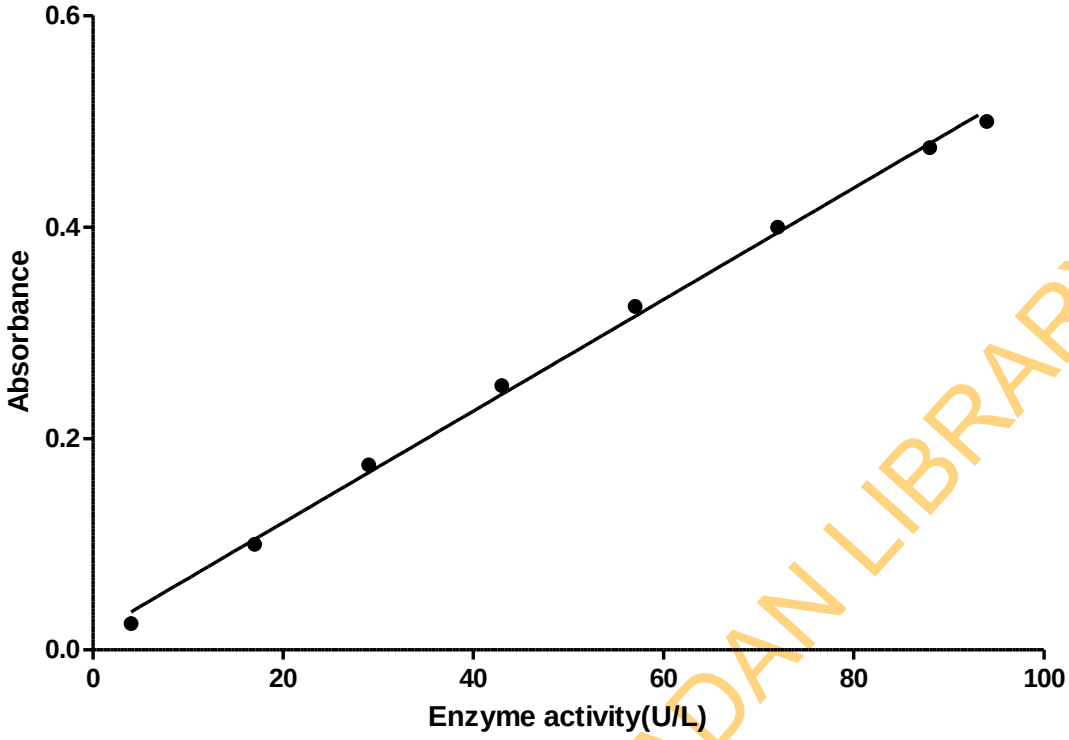


Figure 3.1 Standard plot for serum ALT activity (R^2 = 0.9976).

3.5.2 Serum AST activity determination.

Aspartate aminotransferase (AST) or glutamate oxaloacetate transaminase (GOT) activities were determined using also RANDOX laboratories (UK) reagent kits, based on the method described by Reitman and Frankel (1957).

Principle

α- ketoglutarate + aspartate —AST —→ L- glutamate + oxaloacetate

AST activities were monitored by measuring the absorbance of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine at 546 nm against reagent blank. Enzyme activity units (U/L) were read off from the plots of Absorbance versus Enzyme activity unit (Figure 3.2). Data (Table 3.2) were provided by the manufacturer contained in the manufacturer's instruction manual (AL101) which was supplied along with the reagents.

Reagents

Phosphate buffer	100 mmol/l, pH 7.4
L- aspartate	100 mmol/l
α-ketoglutarate	2.0 mmol/l
2,4-dinitrophenylhydrazine	2.0 mmol/l

Procedure

Wavelength	546 nm
Cuvette	1cm light path
Incubation temperature	37^0C

Contents of the test tubes were as follows:

	Reagent Blank	Test
Sample	-	0.1ml
Buffer	0.5ml	0.5ml

Distilled water	0.1ml	-

The test tubes were shaken and incubated at 37^{0}C for 30 minutes, thereafter 0.5ml of 2,4 – dinitrophenylhydrazine was added to each test tube. The contents of the test tubes were agitated again and allowed to stand for 20 minutes at 20^{0}C after which 5ml 2.5 M NaOH solution was added to each tube. The absorbance of the sample was read after 5 minutes against the reagent blank.

Table 3.2. Data for serum AST activity standard curve.

Absorbance (λ = 546 nm)	Enzyme activity (U/L)
0.020	7
0.040	13
0.060	19
0.080	27
0.010	36
0.120	47
0.140	59
0.160	76

Source: RANDOX Laboratory manual for AST.

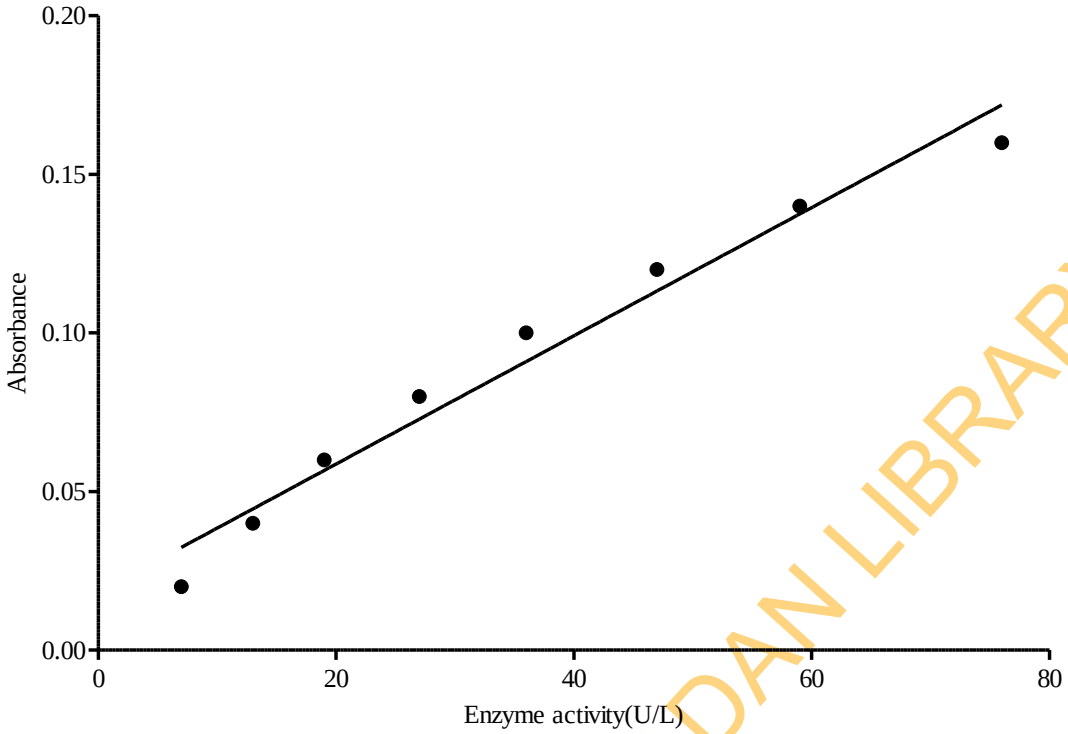


Figure 3.2 Standard plot of serum AST activity (R^2= 0.9696).

3.5.3 Serum quinine oxidase activity determination.

Serum quinine oxidase activity was determined using modification of Baier´s method (1960) by Balazs *et al.,* 1961.

Principle

Quinine is oxidized to quinine carbostyril (2-hydroxy compound of pyridine) which absorbs maximally at 366 nm. One unit of quinine oxidase activity (Baier's unit) is that amount of the enzyme which causes an increase in absorbance of 0.001 under the stated conditions.

Reagents

Quinine sulphate 0.025% in distilled water.

Phosphate buffer solution ($Na_2HPO_4.12H_2O/NaH_2PO_4.12H_2O$), 0.06 M pH 7.2

Distilled water.

Procedure

To 0.2ml.of the rat serum (unhaemolysed) were added 1.5 ml. 0.06 M phosphate buffer pH 7.2 and 1.0 ml. 0.025% quinine sulphate solution. The absorbances of the test solutions were measured against blank at 366 nm immediately and after 60 minutes at the room temperature.

3.5.4 Determination of total protein concentration.

Protein concentration in all samples was determined according to the colorimetric method of Lowry *et al.*, 1951.

Principle

The method combines sensitivities of Biuret reaction in which alkaline Cu^{2+} reacts with the peptide bond to give a deep blue colour with that of Folin-Ciocalteau chemistry in which a complex mixture of phosphomolybdate and phosphotungstate reacts with tyrosine and tryptophan residues to give an intense blue-green colour.

Materials

1. 1% (w/v) $CuSO_4$
2. 2% (w/v) Na tartrate
3. 2% (w/v) Na_2CO_3 in 0.1M NaOH.
4. Folin-Ciocalteau reagent(available commercially)
5. Bovine serum albumin (BSA) standard.
6. Alkaline Copper reagent – prepared by mixing 1ml of 1% (w/v) $CuSO_4$ solution and 1ml. of 2% (w/v) Na tartrate with 98 ml. of 2% (w/v) Na_2CO_3 in 0.1M NaOH solution. The reagent was prepared within 1 hour of use.

Procedure

A total volume of 1.2 ml of the sample was placed in test tube to which 6 ml of alkaline copper reagent was added and mixed immediately, very well. After 10 minutes, 0.3 ml of Folin – Ciocalteau reagent was added and mixed immediately and well. The absorbance of the blue – green coloured sample so obtained was read at 500 nm.

Protein standard curve

A series of BSA concentrations, 2.40, 1.20, 0.60, 0.30, 0.15 and 0.075 mg/ml were prepared in a total volume of 1.2 ml using distilled water to make up to the volume. They were subjected to the protein determination procedure (Table 3.3). A plot of absorbance versus protein concentrations was constructed to obtain a standard curve (Figure 3.3).

Sample protein concentrations were read off from the standard curve.

Table 3.3 Data for protein concentration calibration graph.

Protein concentration (mg/ml)	Absorbance (λ = 500 nm)
0.00	0.000
2.40	0.376
1.20	0.300
0.60	0.233
0.30	0.162
0.15	0.091
0.075	0.056

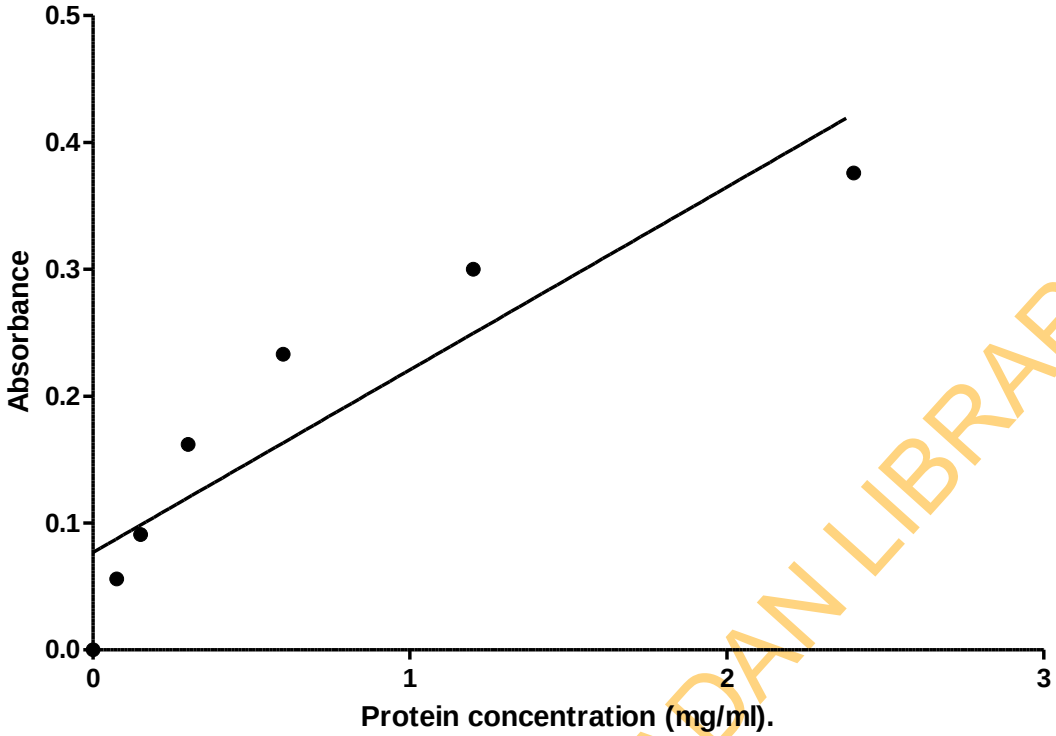


Figure 3.3 Protein concentration calibration curve (R^2 = 0.8359).

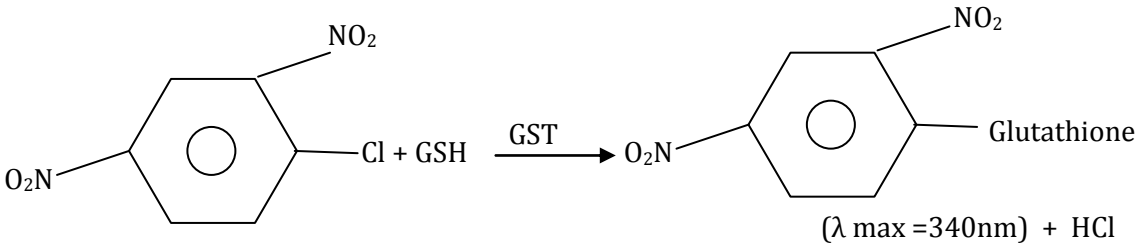
3.5.5 Glutathione S – transferase (GST) activity determination.

GST activities were determined according to the method of Habig *et al.,* 1974. The enzyme catalyzes the conjugation of the endogenous tripeptide, reduced glutathione (GSH), with a large number of structurally diverse electrophilic xenobiotics or their metabolites.

Principle

The principle of the method is according to the reaction ;

1, chloro – 2,4 – dinitrobenzene + GSH —GST→ 2,4 – dinitrophenylglutathione + HCl



The glutathione adduct, 2,4 – dinitrophenyl is determined at 340nm.

Reagents

(i) 1- chloro- 2,4- dinitrobenzene (CDNB) – 30 mM (62 mg to 10 ml ethanol)

(ii) Reduced glutathione (GSH) 30 mM (16 mg to 2 ml water)

(iii) Potassium phosphate buffer, 100 mM pH 6.5

(iv) Post – mitochondrial supernatants from rat livers and kidneys as samples.

Procedure

0.1ml 30 mM GSH solution, 0.1 ml 30 mM CDNB solution, and 2.2 ml 100 mM phosphate buffer, were placed in two test tubes with a micropipette. In one of the test tubes was placed 0.6 ml of the sample and in the other was placed 0.6 ml of 0.25 M sucrose solution (pH 7.4) the medium that was used to prepare the samples. Absorbance was read at 340 nm over 5 minutes on spectrophotometer linked to a graphic printer and the change in absorbance per minute (ΔA/ min) was calculated from the linear portion of the graph.

Enzyme activity (U) = ΔA_{340}/min x 1/9.6 mM

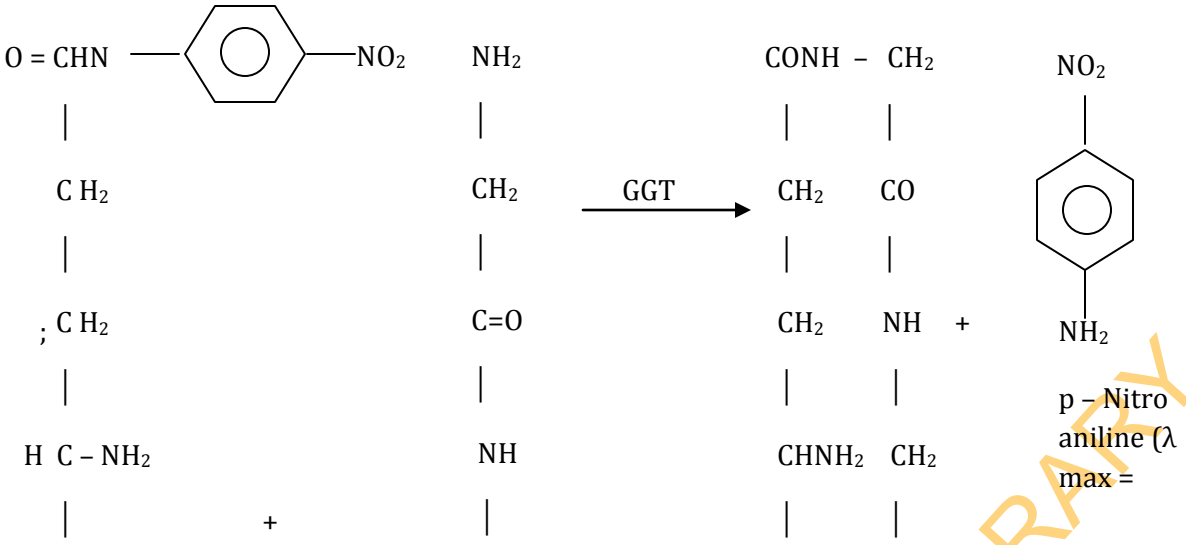
Extinction coefficient for glutathione adduct = 9.6 $mM^{-1}cm^{-1}$

3.5.6 Determination of γ – glutamyltransferase (GGT) activity.

GGT activities in the liver and kidney mitochondrial supernatants were determined using the method of Rosalki and Tarlow (1974).

Principle

The method is based on the transfer of glutamyl residue in the enzyme substrate, γ- glutamyl - p-nitroanilide (GGPNA) by GGT to the acceptor glycylglycine . The p – nitroaniline formed absorbs maximally at 405 nm.



Reagents

(i) Glycylglycine buffer reagent – 115 mmole Tris base/dm^3 and 138 mmole glycylglycine/dm^3 , pH 8.5 – 6.96 g of Tris base and 9.11 g of glycylglycine were dissolved in 400 ml of distilled water. The pH was adjusted to 8.5 at 30^0C by adding 0.5M HCl drop wisely. The buffer solution was then made up to 500 ml. with distilled water.

(ii) GGPNA substrate, 104 mM in 0.5 M HCl – 29.5mg of L – γ – glutamyl – p – nitroanilide monohydrate was dissolved in 1ml. 0.5M HCl , prepared fresh each day.

(iii) Adjustment of buffer glycylglycine concentration – The pH of the buffer on addition of 0.1ml.of the substrate to 0.1ml. of buffer reagent was made to be 8.0 with 0.5M HCl added drop wisely and using pH meter to monitor the pH.

Procedure

2.0 ml of buffer and 0.1 ml. of sample were pipetted into the 1.0 cm cuvette and pre-warmed to 30^0C. A blank was similarly prepared using 0.1 ml of distilled water in place of sample. The reaction was initiated by adding 0.2 ml of the substrate to both the test and the blank and mixed rapidly.

Change in absorbance at 405 nm was monitored at 1 minute intervals for 10 minutes on a spectrophotometer linked to a computerized graphic printer.

Calculation:

Enzyme activity (U/l) = $\Delta A/min \times 10^3/9.87 \times 2.3/0.1 = \Delta A/min \times 2330$

Where $\Delta A/min$ = absorbance change/min = observed rate of reaction

9.87 x litre x $mmole^{-1}$ x cm^{-1} = millimolar extinction coefficient for p - nitroaniline

2.3 ml = total reaction volume; 0.1 ml = sample volume.

3.5.7 Determination of glutamate dehydrogenase (GDH) activity.

GDH activity was determined in rat brain mitochondrial preparations by the method of Smith *et al.,*1975.

Principle

The enzyme catalyzes reductive amination of α-ketoglutarate, using NADH as co-enzyme forming glutamate.

$$\alpha - \text{ketoglutarate} + NH_3 + NADH + H^+ \rightleftharpoons \text{Glutamate} + H_2O + NAD^+$$

The reaction favours glutamate synthesis. The disappearance of NADH is measured spectrophotometrically at 340 nm.

Reagents

A. Potassium phosphate buffer 50 mM pH 7.4 : 50 mM K_2HPO_4 and 50 mM KH_2PO_4 solutions were prepared and mixed and adjusted to pH 7.4.

B. Imidazole-HCl buffer – 58 mM pH 7.3: 3.95 g of imidazole was dissolved in 800 ml of distilled water. The pH was adjusted to 7.3 with concentrated HCl added drop wisely. The buffer was made up with distilled water to $1dm^3$.

C. α – ketoglutarate solution 0.13 M pH 6.5 ; 475 mg of α – ketoglutarate was dissolved in 8 ml of distilled water. The pH was adjusted to 6.5 with 2 M NaOH added in drops, monitored with a pH meter. The solution was made up with distilled water to 25 ml.

D. NADH solution, 4mM. : 14mg of NADH – Na was dissolved in 5 ml of 50 mM potassium phosphate buffer pH 7.4 (**A**).

E. Ammonium acetate, 1.6 M ; EDTA, 4 mM solution – 123 g of ammonium acetate and 14.9 g of EDTA-Na_2 were dissolved in 8 ml of distilled water and diluted with same to 10 ml .

Procedure

Reagents B – 2.4 ml

C – 0.3ml

D – 0.1ml

E – 0.1ml

The blank was prepared by replacing sample with equal volume of distilled water.

The contents were equilibrated at 25^0C for 5 minutes and 0.1ml of sample was added and mixed properly. Decrease in absorbance was read at 340 nm.

Calculation:

Enzyme activity (U/l) = $\Delta A/min \times 3.0ml/0.1ml \times 10^3/6.22$

6.22 = millimolar extinction coefficient of NADH at 340nm.

One unit of enzyme activity is defined as the amount of enzyme which produces 1µmole of NAD^+ per minute at 25^oC and pH 7.3 under the stated conditions above.

3.5.8 Malondialdehyde concentration determination.

Malondialdehyde concentration was estimated in the rat liver, kidney and brain tissues by the method of Yuda *et al.* **,** 1991.

Principle

2 – Thiobarbituric acid (TBA) reacts with malondialdehyde in an acidic medium to produce a pink coloured complex which absorbs maximally at 532 nm. The coloured complex is extractable into organic solvents such as butanol.

Reagents

(i) Tris – HCl buffer 50 mM pH 7.2

(ii) TBA – 0.6%

(iii) n–Butanol

Procedure

0.5 ml of the prepared sample was placed in an incubator with 0.1 ml of Tris – HCl buffer pH 7.2 at 37^0C. After incubation, 9 ml of distilled water and 2 ml of 0.6% TBA were added to the solution and shaken vigorously. The mixture was heated for 30 minutes in a boiling water bath. The solution was allowed to cool naturally to the laboratory temperature and 5 ml of n – butanol was added and again shaken properly. The pink n – butanol layer was separated by centrifugation at 1000xg for 10 minutes and the malondialdehyde production was measured at 532 nm against blank.(Wong *et al.*, 1987). Extinction coefficient of malondialdehyde = $1.56 \times 10^5 M^{-1}cm^{-1}$

Calculation : A_{532} /1.56 x 10^{-5} x 10^{-3} mole MDA/mg protein. (10^{-3} = conversion factor to ml, noting that extinction coefficient is expressed in mol/dm^3).

3.5.9 Superoxide dismutase (SOD) activity determination

SOD activity was determined in the samples by the method of Misra and Fridovich (1972).

Principle

The principle of the assay is the inhibition by SOD of autooxidation of epinephrine to adrenochrome at high pH.

Reagent mixture

Epinephrine - 3 x 10^{-4} M,

EDTA - 1 x 10^{-4} M ,

Na_2CO_3 solution - 0.05 M pH 10.2,

The mixture was dissolved in a total volume of 3 ml. of deionized water. The reagent mixture was added to 0.2 ml sample and equilibrated at 30^0C. Absorbance was read on spectrophotometer at 480 nm against reagent blank. The decrease in absorbance was monitored for 5 minutes at 30s intervals on the spectrophotometer linked to a computerized graphic printer.

One unit of SOD activity (U) is the amount of enzyme which caused 50% inhibition of epinephrine autooxidation to adrenochrome under the assay condition.

Calculation

Activity U = ΔA_{480} /min × 1/e x d.f. /50% (Misra and Fridovich, 1972).

Where ΔA_{480} = absorbance change at 480nm. ; d.f. = dilution factor = 15

e = extinction coefficient of adrenochrome = 4.02 x 10^3 $M^{-1}cm^{-1}$

3.5.10 Urine trehalase activity determination

Principle

Trehalase hydrolyses trehalose to two glucose molecules. The glucose thus formed is determined spectrophotometrically. The observed change (increase) in concentration of glucose in the unknown is compared to the control to give the trehalase activity (Sasai – Takedatsu *et al.,* 1995).

Reagents

Trehalose solution - 0.25 M in phosphate buffer.

Buffer ($Na_2HPO_4.12H_2O$/$NaH_2PO_4.12H_2O$) - 1.0 M pH 6.2

Glucose kit (RANDOX) Catalogue Number GL 364.

Procedure

0.1 ml of the trehalose solution was added to 0.9 ml of urine sample. The control contained 0.1 ml distilled water and 0.9 ml of urine. Both tests were incubated at 37^0C for 2 hours. The glucose thus formed was determined using RANDOX reagent kit based on the principle of glucose oxidation by glucose oxidase. The H_2O_2 so produced following glucose oxidase action reacts with 4-aminophenazone in the presence of phenol catalyzed by peroxidase to form quinoneimine, the absorbance of which was read at 500 nm against reagent blank. Sample glucose concentration was calculated by comparing the absorbance (A) of the sample against the standard glucose solution;

Glucose concentration (mM) = A(sample)/A(standard) x glucose standard concentration. The observed increase in the concentration of glucose gives the trehalase activity in micromoles of glucose /hr/litre.

3.5.11 Colorimetric estimation of creatinine

Urine creatinine was determined by a method based on Jaffe reaction as described by Plummer (1971).

Principle

Creatinine in alkaline picric acid solution forms a red tautomer of creatinine picrate which absorbs maximally at 530 nm.

Reagents

Saturated aqueous solution of picric acid.

1M NaOH.

Standard creatinine solution 2 mg/100ml

Procedure

1ml of sample was placed in 100 ml volumetric flask to which 1ml of 1M NaOH and 2 ml. of saturated picric acid solution was added. The solution developed was mixed thoroughly and stood

for 10 minutes. It was made up to the mark with distilled water. A series of standard creatinine solutions were prepared (Table 3.4) for the construction of calibration curve (Figure 3.4). The concentrations of the samples were read off from the graph.

Table 3.4. Data for creatinine concentration calibration graph.

Creatinine concentration (mg/ml)	Absorbance (at 530nm)
0.0	0.000
1.0	0.219
1.2	0.251

1.4	0.284
1.6	0.316
1.8	0.348
2.0	0.381

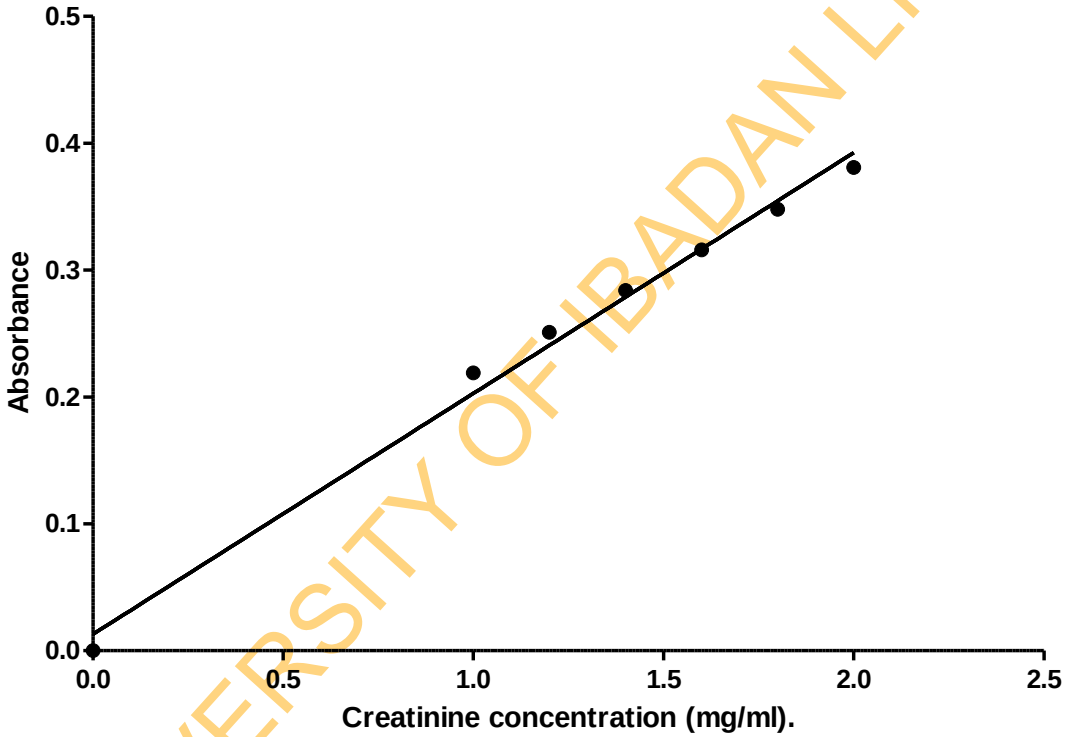


Figure 3.4 Creatinine concentration calibration graph (R^2 =0. 9922).

3.5.12 Urine magnesium ion concentration determination

Principle

The urine sample diluted to a total volume of 2 ml. with deionized water to which 10 ml. conc. HCl was added to prevent precipitation of magnesium (Alcock *et al.*, 1960) was analyzed by atomic absorption spectrophotometer with Deuterium lamp. The control consisted of 2 ml. deionized water and 10 ml. conc. HCl . The standard Mg ion solution was provided by the manufacturer of the equipment.

3.6 Stastistical Analyses

One - way Analysis of Variance (ANOVA) with Bonferroni multiple comparisons paired t - test for pre- and post - treatment sample data and Dunnett multiple comparisons

unpaired t - test for all other sample data were employed for the statistical analyses . Linear regression analyses were carried out where appropriate. The software was GraphPadInStat 3, 2009 edition.

CHAPTER FOUR

RESULTS

The results of the *in vitro* experiment using chicken eggs embryonation procedure were as presented in Table 4.1 The response dose (RD_{50}), the dose toxic to 50% of the embryos calculated from the data in Table 4.1 was 427mg/kg using the normograph of Tint and Gillen (1961).

Table 4.1 *In vitro* injection of lamivudine into the embryonated eggsof *Gallus domesticus*.

Lamivudine dose(mg/kg)	No of fertile eggs	No of unhatched/dead embryos
0.0	30	0
4.0	30	2
20.0	30	5
100.0	30	8
500.0	30	21
2500.0	30	30

In the animal experiments, no mortality attributable to the drug was recorded among the rats during both single and repeated dosing experiments. Physical examination of the experimental animals on single doses did not reveal any toxicity signs. Those animals on repeated doses were found less active and towards the end of the dosing periods became aggressive.All the animals were observed to have gained weight (Tables 4.2 &4.3).Body – weight gain among the classes of the animals on single dosing followed no specific pattern (Table 4.2).However, weight-gain was noticeably reduced in the rats exposed to repeated oral doses than those on single doses (Tables 4.2 & 4.3). Body-weight change among the rats on single doses were found to be fairly positively correlated (r = +0.5384) with dose (Table 4.2), whereas among rats on repeated exposure, the change was highly negatively correlated (r = - 0.8222) (Table 4.3).

Table4.2. Body -weight change of rats following single oral doses of lamivudine.#

Lamivudine dose (mg/kg)	Initial weight(g)	Final weight(g)	Weight change (%)
0.0	151.7 ± 4.04	191.8 ± 6.86	+26.3 ± 1.46
4.0	160.2 ± 3.63	205.8 ± 5.71	+28.4 ± 0.94
20.0	141.4 ± 4.49	178.6 ± 7.92	+26 ± 2.08
100.0	108.9 ± 3.27	135.8 ± 5.02	+24.6 ± 1.02
500.0	140.2 ± 5.06	181.0 ± 8.65	+29 ± 2.61

Values are Means ± Standard Error Mean (SEM). 6 rats/group

Table 4.3. Body-weight change of rats following repeated oral doses of lamivudine for 45 days[#]

Lamivudine dose (mg/kg/day)	Initial weight (g)	Final weight (g)	Weight change (%)
0.0	158.7 ± 2.62	208.2 ± 4.74	+31.2 ± 1.14
4.0	140.4 ± 2.45	177.7 ± 6.04†	+28 ± 1.14
20.0	117 ± 4.04	142.4 ± 7.14†	+21.3 ± 2.12
100.0	163.4 ± 3.80	197.9 ± 6.41†	+21 ± 1.25
500.0	150.1 ± 4.78	172.1 ± 7.31†	+14.5 ± 1.47

#Values are means ± S.E.M † Means significantly different from controls $P<0.05$ otherwise $p>0.05$ (Paired t-test)

The results of the analyses on sera of rats on single doses were as presented in Table 4.4which appeared to bear no relationship with the exposure to the drug at any of the doses. None of the parameters, the enzyme activities and protein concentration, showed significant change ($p > 0.05$) against the drug either when compared with the control group or with 4mg/kg group.The results of the analyses on sera of the rats placed on repeated dosing presented in Table 4.5however showed increase in the respective activities of the enzymes namely, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), and quinine oxidase, all statistically significant ($p<0.05$) at 500mg 3TC/kg body-weight when compared with the control but were respectivelynot statistically significant ($p>0.05$) when compared with 4mg/kg.Serum total protein was observed generallyto be depressed in the rats. The depression was highly significant ($p<0.01$) at 500mg/kg when compared with both the control and 4mg/kg (Table 4.5).

Table 4.4. Serum enzymes activities and total protein concentration of rats following single oral doses of lamivudine(3TC)[#]

Lamivudine dose (mg/kg)	ALT (I.U)	AST (I.U)	GGT (I.U)	Total Protein (mg/ml)
0.0	23.5045 ± 0.934	45.8278±1.043	12.7361 ±0.431	5.2428 ±0.546
4.0	23.6383 ±1.217	46.2082±1.237	12.9991 ±0.607	4.5099 ±0.462
20.0	22.3841 ±1.094	45.3126 ±1.036	12.8623±0.597	5.5711 ±0.244
100.0	24.7958±1.656	45.2846±1.281	13.1524±0.466	5.6646 ±0.487
500.0	25.9506 ±1.560	43.3804 ±1.185	13.3442±0.614	5.5129 ±0.690

#Values are Means ± S.E.M. Means not significantly different from the control ($p>0.05$).

Table 4.5. Serum enzymes and protein of rats following daily oral doses of 3TC for 45 days#

Lamivudine dose (mg/kg/day)	ALT(I.U)	AST(I.U)	GGT(I.U)	Q.O(B.U)	Total Protein (mg/ml)
0.0	23.3087 ±1.507	42.5710 ±1.960	14.3075 ±0.719	4.9017 ± 1.026	6.134 ±0.61
4.0	25.0575 ±1.306	43.1957 ±1.175	13.8633 ± 0.812	5.7375 ±1.325	6.186 ±0.35
20.0	27.7433 ±1.99	43.4660 ±1.377	15.2150 ± 1.121	7.7660 ±1.155	5.325 ±0.34
100.0	28.4802 ±2.529	44.7498 ±2.336	15.8780 ± 2.653	8.8580 ±1.582	4.791 ± 0.45
500.0	33.1847 ±3.957†	56.4205 ±7.171†	16.2613 ±1.778†	10.0650 ±1.689†	3.945 ±0.20‡

#Values are Means ± S.E.M Mean significantly different from the control † $p<0.05$; ‡$p<0.01$, otherwise $p>0.05$; Q.O = Quinine oxidase; B.U.= Baier's Unit.

In the post-mitochondrial supernatant of the liver, both GGT and GST specific activities in the rats were found to increase significantly ($p<0.05$) at≥20mg/kg (Table 4.6) when compared with both the control and 4mg/kg (Table4.6).MDA concentration and SOD specific activity wereboth increased in the rat liver by the applied drug. While the MDA concentration was significantly ($p<0.05$) increased at ≥100mg/kg, SOD specific activity was significantly ($p<0.01$) increased at ≥20mg/kg when compared with the control (Table 4.7).

Table4.6 Hepatic tissue GGT and GST specific activities ofrats following daily oraldoses of 3TC for 45days[#]

Lamivudine doses (mg/kg/day)	Hepatic GGT specific activity (U/mg protein).	Hepatic GST specific activity (x10 $^{-2}$ U/mg protein).
0.0	3.5487 ± 0.511	8.7833± 0.746
4.0	6.1883 ± 1.161	11.4250 ± 1.432
20.0	10.2442 ± 1.974†	14.9767 ± 1.706†
100.0	12.4675 ± 1.171$^{*\Delta}$	15.8083 ± 1.713†
500.0	15.3658 ± 2.239*‡	18.2333 ± 2.094$^{*\Delta}$

Values are Means± S.E.M.. Mean significantly different from the control †$p<0.05$,*$p<0.01$; from 4mg/kg, $\Delta p<0.05$, ‡$p<0.01$,otherwise $p>0.05$ when compared with the control.

Table 4.7. Liver tissue MDA concentration and SOD specific activity of rats following daily oral doses of 3TC for 45days#

Lamivudine dose (mg/kg/day).	Hepatic MDA concentration (nmole/mg protein).	Hepatic SOD specific activity (U/mg protein).
0.0	0.0571 ± 0.011	9.9583 ± 0.698
4.0	0.0730 ± 0.013	12.0100 ± 0.927
20.0	0.1560 ± 0.032	14.7017 ± 1.491*
100.0	0.1647 ± 0.029†	14.7383 ± 1.202*
500.0	0.2228 ± 0.041*‡	16.5267 ± 1.320$^{*\Delta}$

#Values are Means ± S.E.M. Mean significantly different from (i) the control,†$p<0.05$

* $p<0.01$, (ii) 4mg/kg , Δ$p<0.05$, ‡$p<0.01$ otherwise $p>0.05$ when compared with the control.

Overnight (12 hours) urine samples of the experimental rats prior to and after dosing were analyzed as test of renal integrity following lamivudine administration. Paired comparisons of the parameters in the urine samples namely, trehalase activity, magnesium and protein concentrations, were undertaken between pre-treatment urine and post-treatment urine in view of possible falsification due to individual differences. These parameters were expressed in terms of individual urine creatinine concentration to reduce the effect of diurnal/circadian changes in the values of the urinary parameters. Post- treatment urinary trehalase activity, protein, as well as

magnesium ions concentrations respectively expressed as urinary creatinine, were found to increase in rats exposed to both single and repeated doses of the drug (Tables 4.8 &4.9). However, paired comparisons between the pre- and post- treatment urinary parameters in rats exposed to the singleoral doses of the drug did not show statistical significance ($p > 0.05$) at any of the doses (Table 4.8). But similar comparisons in the rats on daily dosing (for 45days) were found to be statistically significant at ≥100mg/kg. The effect of the drug on the urine protein concentration at 100mg/kg appeared less pronounced than on the other parameters at the same dose (compare $p<0.05$ to $p<0.01$)(Table 4.9). Trehalase activitycorrelated positively with protein/creatinine ratio. ($r = +0.95$) (Table 4.9).

Table 4.8. Renal toxicity in the rats orally administered single doses of 3TC.*

Lamivudine dose (mg/kg)	Urine Trehalase activity /Creatinine conc. (U/mg/ml)	Urine Mg^{2+} conc./Creatinine conc. x 10^{-3}	Urine Protein conc./Creatinine conc.

0.0	25.8327 ± 3.082	5.0547 ± 0.806	1.3094 ± 0.111
4.0	25.6978 ± 4.259	5.6000 ± 0.450	1.3432 ± 0.066
20.0	28.9336 ± 3.810	4.9283 ± 0.731	1.3069 ± 0.010
100.0	24.0715 ± 1.967	4.6002 ± 0.419	1.0860 ± 0.059
500.0	29.5480 ± 2.318	5.2386 ± 0.749	1.1438 ± 0.215

*Values are Means ± S.E.M. Paired t-test - Means not significantly different from the corresponding pre-treatments, $p>0.05$. U= µmole glucose/hr/litre.

Table 4.9. Renal toxicity in the rats following oral administration of lamivudine daily for 45days.#

Lamivudine Dose (mg/kg/day)	Urine Trehalase activity /Creatinine	Urine Mg^{2+}conc/Creatinine	Urine Protein conc/Creatinineconc*

	conc (U/mg/ml)	conc x 10^{-3}*	
0.0	157.7937 ± 18.608	4.7913 ± 0.490	1.3863 ± 0.138
4.0	130.2916± 19.832	3.2382 ± 0.103	1.0819 ± 0.030
20.0	200.9225± 34.435	5.1680 ± 0.654	1.4801 ± 0.199
100.0	324.0727± 35.332‡	8.5656 ± 0.403‡	2.9046 ± 0.290†
500.0	386.2056± 70.050‡	9.1946 ± 0.637‡	3.2274 ± 0.307‡

#Values are Means ± S.E.M. Paired t-test. Mean significantly different from the corresponding pre-treatment. †$p<0.05$, ‡$p<0.01$ otherwise $p>0.05$ when compared with the control.*$mgml^{-1}/mgml^{-1}$;

U=µmole glucose/hr/litre.

Inratsrepeatedly exposed to the drug, analysis of renal tissuepost mitochondrial supernatants showed that the mean specific activity of γ – glutamyltransferase (GGT) declined with increasing dose (Table 4.10). The decline was however not significant ($p>0.05$)whether compared with the control or with 4mg/kg at any of the doses, this is at variance with what obtained in the liver where as much a dose as 20mg/kg produced significant effect.The GST specific activity in the

renal tissue increased with dose as was obtained in the liver. This increase was statistically significant ($p < 0.01$) at ≥100mg/kg ($p<0.05$ at 20mg/kg for the liver) when compared with both the control and the 4mg/kg group (Table4.10).

Table 4.10. Renal tissue GGT and GST specific activities in rats following daily oral doses of 3TC for 45days[#]

Lamivudine doses (mg/kg/day).	GGT specific activity (U/mg protein).	Renal GST specific activity ($x10^{-2}$ U/mg protein).

0.0	149.4350 ± 11.208	1.7433± 0.234
4.0	142.640 ± 22.952	1.9550 ± 0.057
20.0	139.0733 ± 7.582	2.3567 ± 0.255
100.0	135.820 ± 9.380	3.1450 ± 0.121*‡
500.0	128.4483 ± 19.216	3.5717 ± 0.231*‡

. #Values are Means± S.E.M. Mean significantly different from the control †p<0.05, *p<0.01 ; from 4mg/kg ,Δ p<0.05, ‡p<0.01, otherwise p>0.05 when compared with the control.

As in the liver tissue, both malondialdehyde (MDA) concentration and superoxide dismutase (SOD) specific activity in the rat renal tissue increased as the dose(Table4.11). The MDA concentration was not significant ($p>0.05$) at any of the doses when compared with the control but significant ($p<0.05$ and $p<0.01$) at 100mg/kg and 500mg/kg respectively when compared with 4mg/kg. The increased SOD specific activity was significant ($p<0.05$) at ≥100mg/kg when compared with the control or with 4mg/kg (Table4.11).

Table 4.11. Renal tissue malondialdehyde(MDA) concentration and SOD specific activity in rats following daily oral doses of 3TC for 45days#

Lamivudine dose (mg/Kg/day).	Renal MDA concentration (nmole/mg protein).	Renal SOD specific activity (U/mg protein).

0.0	0.5570 ± 0.094	6.9157 ± 0.569
4.0	0.7572 ± 0.127	9.5774 ± 1.608
20.0	0.9879 ± 0.106	12.003 ± 1.938
100.0	1.6828 ± 0.290$^{\Delta}$	13.9394 ± 1.641†
500.0	2.2004 ± 0.418‡	17.5580 ± 1.475*‡

#Values are means® ± S.E.M..Mean significantly different from the control,† $p<0.05$,

* $p<0.01$;from 4mg/Kg , Δ $p<0.05$ ‡$p<0.01$, otherwise $p>0.05$ when compared with the control.

In the rat brain homogenates, MDA concentration was found to increase with dose while SOD specific activity declined. However, both changes were not statistically significant ($p>0.05$) whether compared with the controls or with 4mg/kg (Table4.12). The results were a departure from what obtained in the liver (Table 4.7) and the kidney (Table 4.11) where the applied drug produced significant effects.

The specific activity of glutamate dehydrogenase in the brain homogenates increased as the dose, but the increase as with SOD, was also statistically not significant($p>0.05$) at any of the doses when compared with the control or with 4mg/kg (Table 4.12).

Table4.12 . Brain tissue MDA concentration, superoxide dismutase (SOD) and glutamate dehydrogenase (GDH) activities in rats following repeated oral doses of 3TC for 45days *

Lamivudine doses (mg/kg/day)	MDA concentration (nmole/mg protein)	SOD specific activity (U/mg protein)	GDH specific activity ($x10^{-3}$ U/mg protein).

0.0	0.8423 ± 0.173	10.644 ± 1.341	8.8400 ± 1.512
4.0	0.8509 ± 0.157	9.4821 ± 1.644	8.9717 ± 0.157
20.0	1.1561 ± 0.183	8.9892 ± 0.825	10.5650 ± 1.943
100.0	1.3154 ± 0.170	8.4805 ± 0.245	11.4383 ± 1.326
500.0	1.4479 ± 0.185	7.2297 ± 0.227	13.9333 ± 0.975

*Values are Means ± S.E M. Data not significant ($p>0.05$) when compared with the control.

Increased tissue-weight/body-weight ratio was observed for both the kidney and the liver at all the dose levels, a departure from what obtained for the brain (Table 4.13). Both the kidney-and liver -weight/body-weight ratios were found tobe significantly increased($p<0.01$) at 500mg/kg when compared with both the control and 4mg/kg. The observed reduction in brain-weight/body-weight ratio was not statistically significant ($p>0.05$) at any of the doses when compared with the control or with 4mg/kg.

Table 4.13. Tissue-weight/Body-weight change in the rats following daily oral doses of 3TC for 45days#

Lamivudine dose (mg/kg/day).	Kidney/Body- weight ratio.	Liver/Body-weight ratio.	Brain /Body-weight ratio.
0.0	0.6100 ± 0.019	3.4683 ± 0.051	0.5983 ± 0.022
4.0	0.5883± 0.017	3.4950 ± 0.077	0.5783 ± 0.019
20.0	0.6317 ± 0.015	3.5900 ± 0.065	0.5633 ± 0.022
100.0	0.6617 ± 0.012$^{\Delta}$	3.6200 ± 0.055	0.5617 ± 0.027
500.0	0.7000 ± 0.015*‡	4.0067 ± 0.102*‡	0.5567 ± 0.014

#Values are Means ± S.E.M x 10^{-2} mg/g. Mean significantly different,from the control *$p<0.01$; from 4mg/kg ,Δ$p<0.05$, ‡$p<0.01$, otherwise $p>0.05$ when compared with the control.

Histopathological studies carried out on the chick (*Gallus domesticus*) embryos recorded various lesions in the livers starting from very sparse lymphoid aggregates occasioned by injection of at least 4mg/kg to dense multifocal lymphoid cell population in the sinusoid.

Figures 4.1 to 4.10 show the photomicrographs of histologic preparations of 10 days old embryos of *Gallus domesticus* injected with the indicated concentrations of lamivudine (3TC).

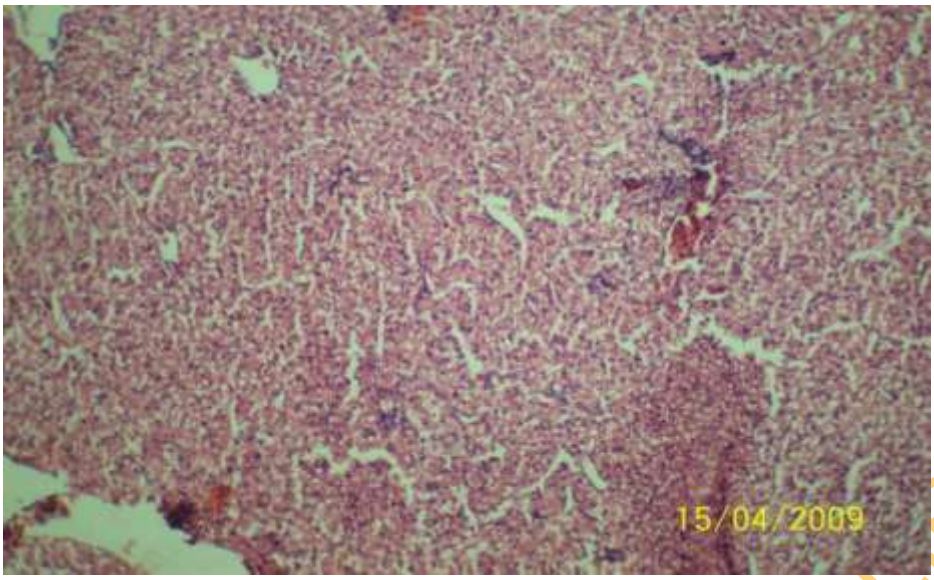


Figure 4.1.

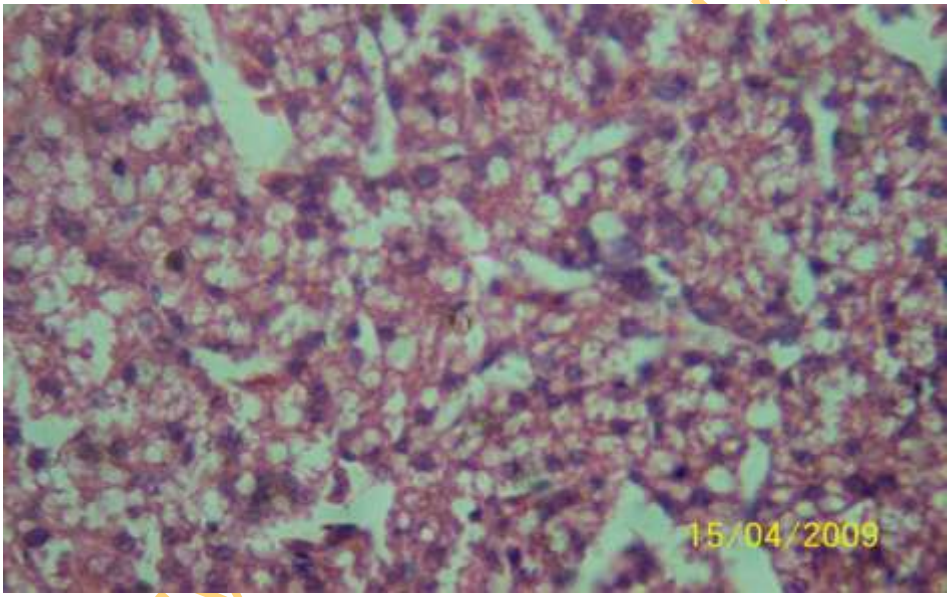


Figure 4.2.

Figures 4.1.&4.2. Livers (H & E ×100) of chick embryos following injection of 0.5ml normal saline(the control) showing no visible lesion.

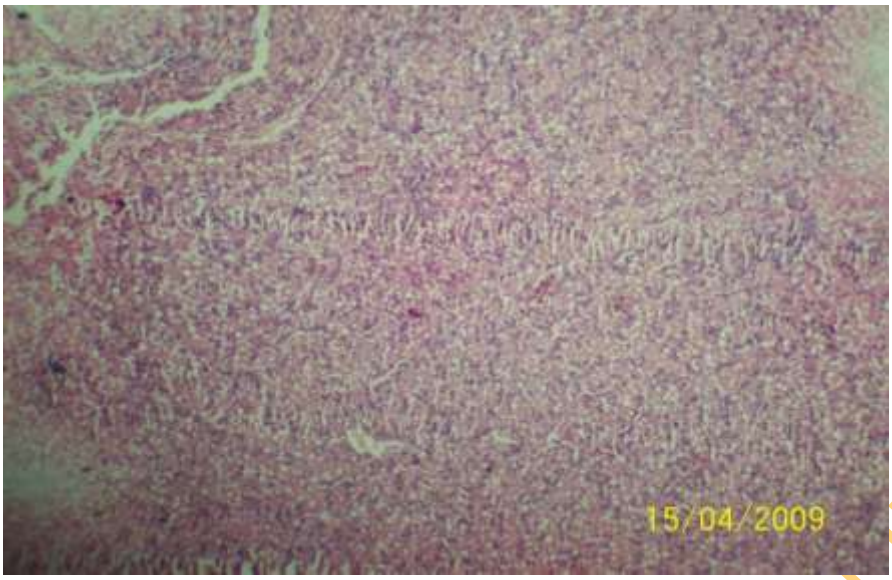


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Figure4.3.

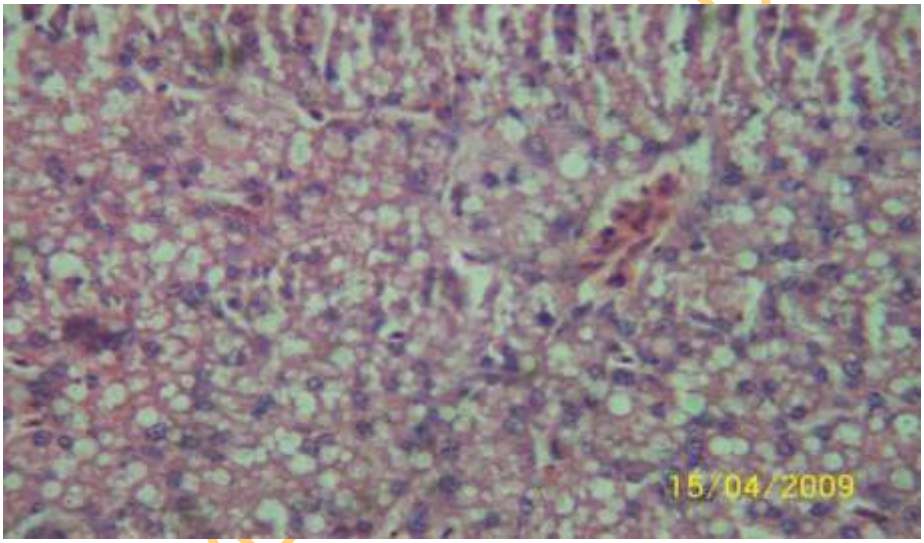

Figure 4.4.

Figures 4.3&4.4. Livers (H& E ×100)of the chick embryos following injection of 4mg 3TC/kg body weight showing lymphoid aggregates, very sparse.

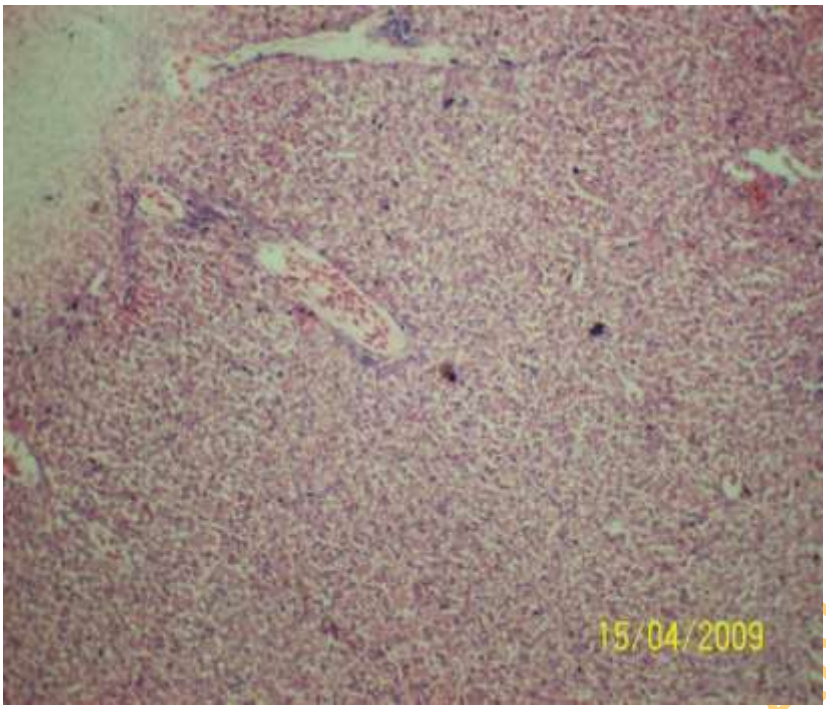

Figure 4.5.

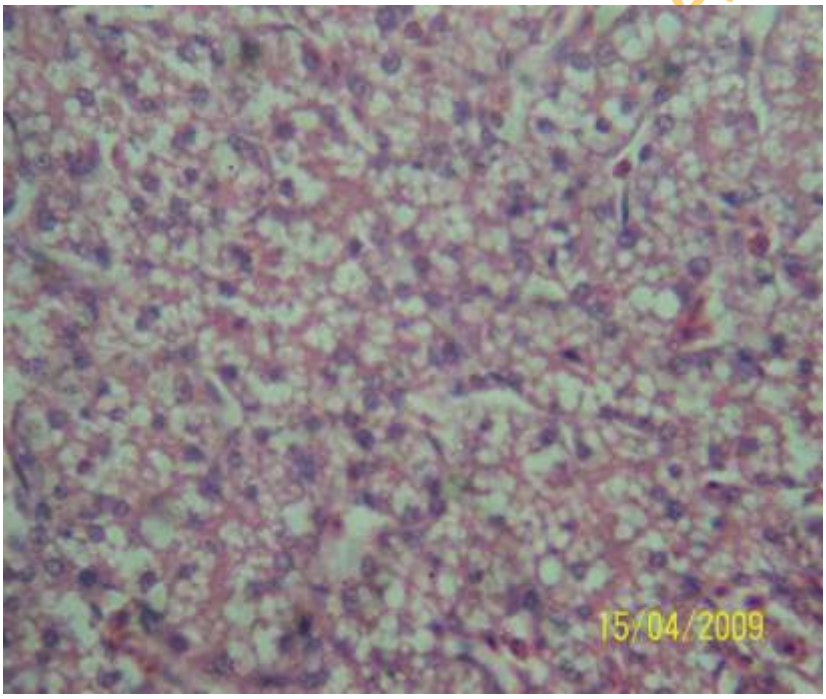

Figure 4.6.

Figures4.5 &4.6. Livers (H&E ×100) of the chick embryos following injection of 20mg 3TC/kg body weight showing lymphoid aggregates very sparse and very mild portal congestion.

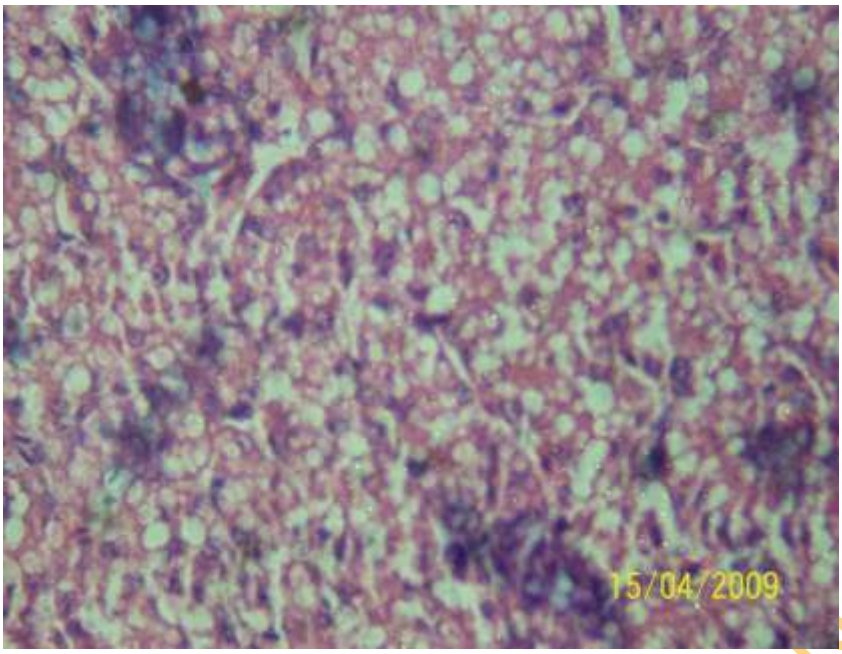

Figure 4.7.

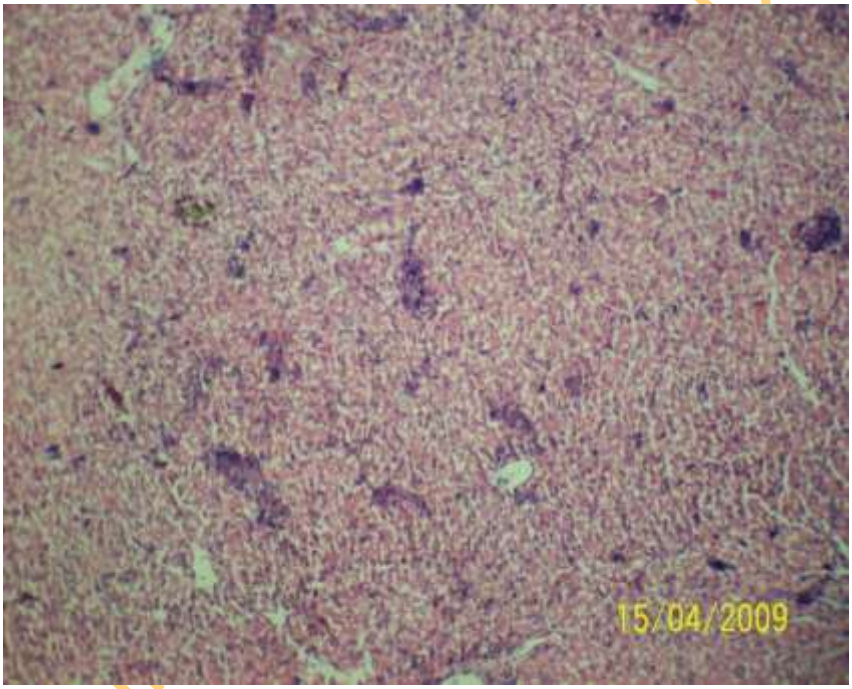

Figure 4.8.

Figures 4.7 &4.8. Livers (H&E × 100)of chick embryos following injection of 100mg 3TC/kg body weight showing moderately dense lymphoidcompartment.

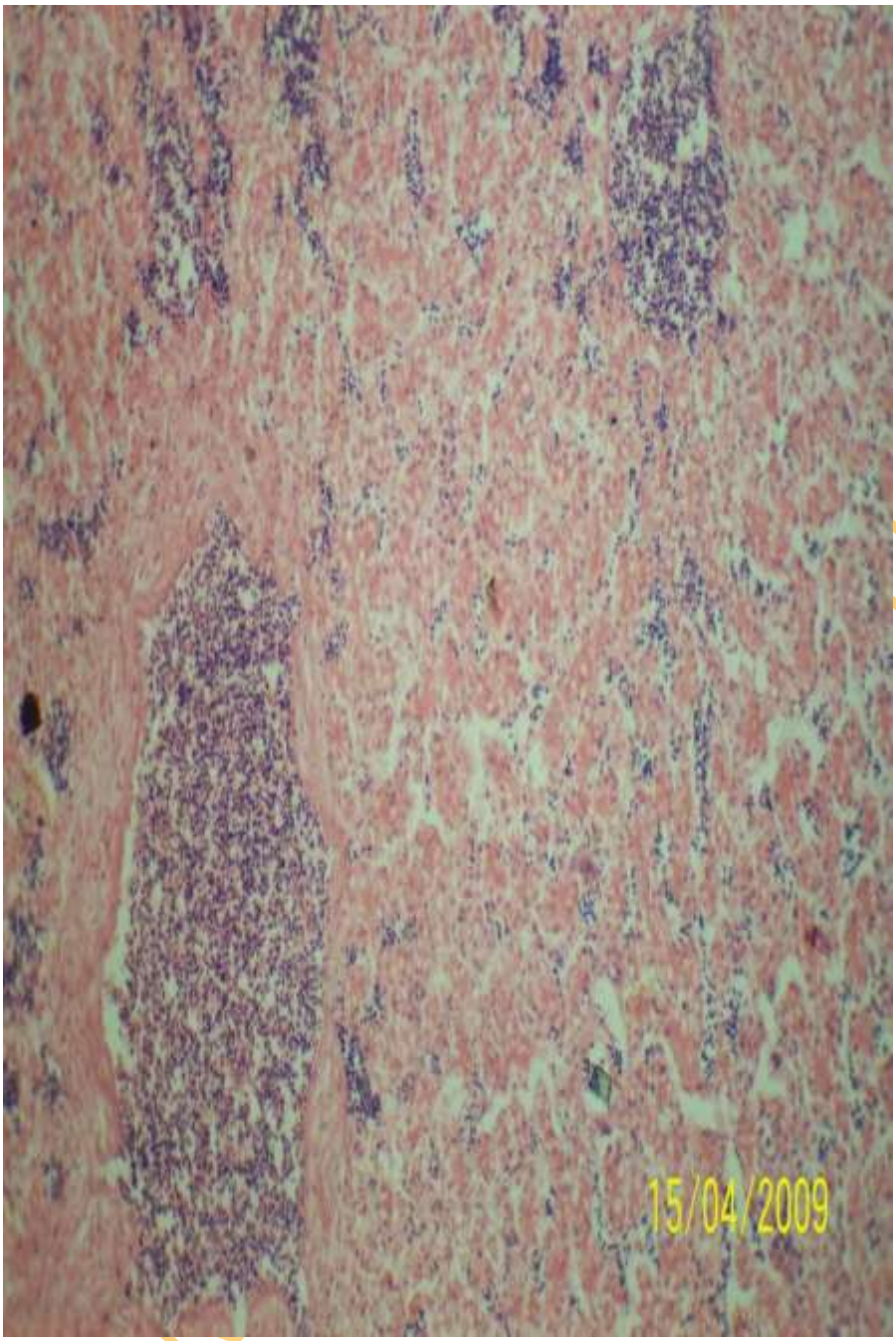

Figure4.9.Liver(H&E × 100) of chick embryo following injection of 500mg 3TC/kg body weight showing highly dense multifocal lymphoidcell population in the sinusoid.

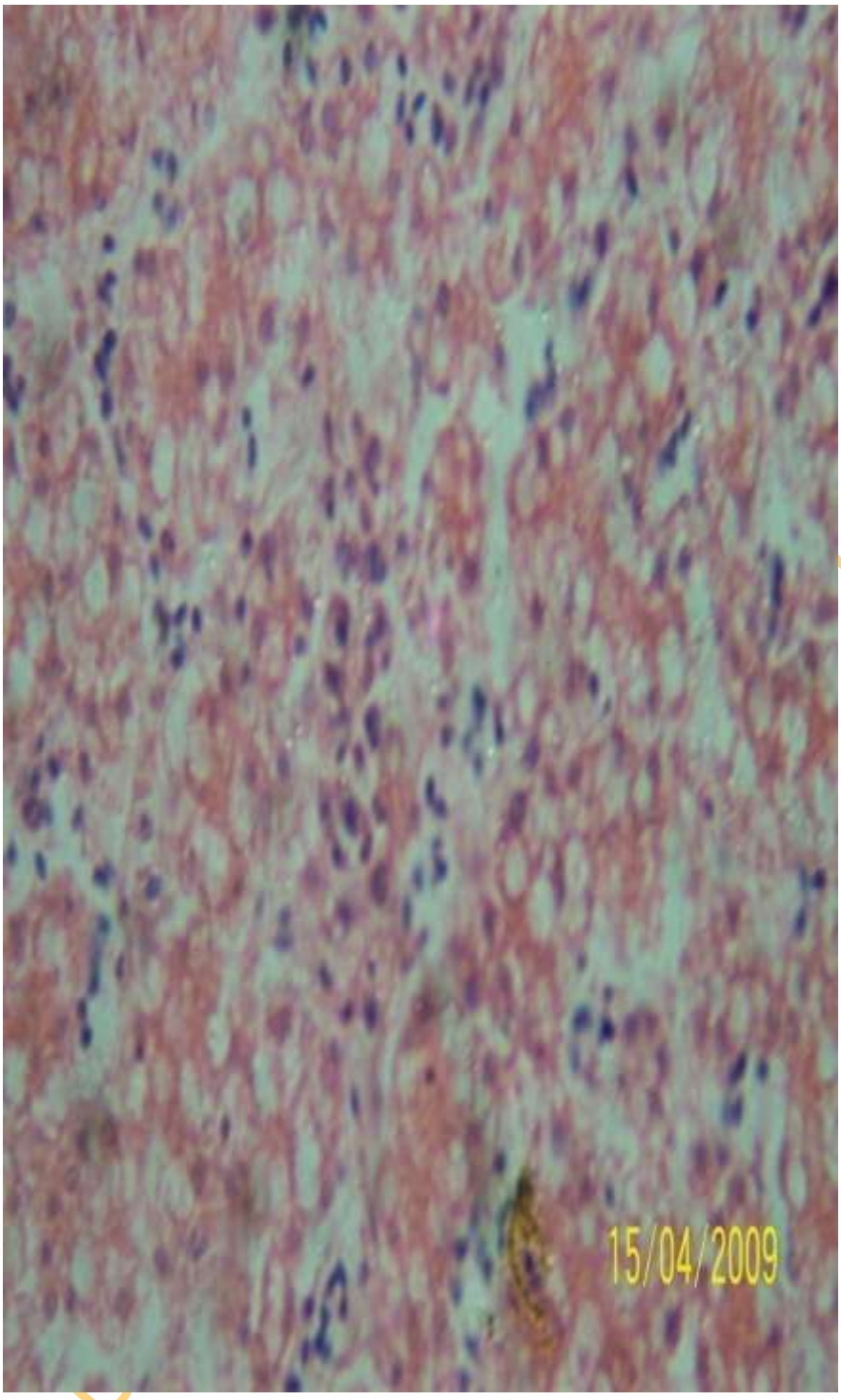

Figure 4.10. Liver (H & E x 100) of chick embryo following injection of 2500mg 3TC/kg body weight showing highly dense multifocal lymphoid cell population in the sinusoid.

The livers and the kidneys of the rats repeatedly exposed to the drug orally, showed no visible lesion at 4mg/kg (human therapeutic dose) (Figure4.13). However, 20mg/kg which produced congestions and hydropic degeneration of hepatocytes in the liver failed to produce any visible

lesion in the kidney tissue (Figures 4.17and 4.18). Lesion in the renal tissue (cortical congestion) induced by the drug was first recorded at 100mg/kg. The intensity of lesion in both tissues appeared to be dose-dependent.

Figures 4.11 to 4.20 show photomicrographs of the liver and kidney tissues of the rats following daily oral doses respectively of 0.2ml of distilled water and lamivudine(3TC)at different concentrations for 45 days.

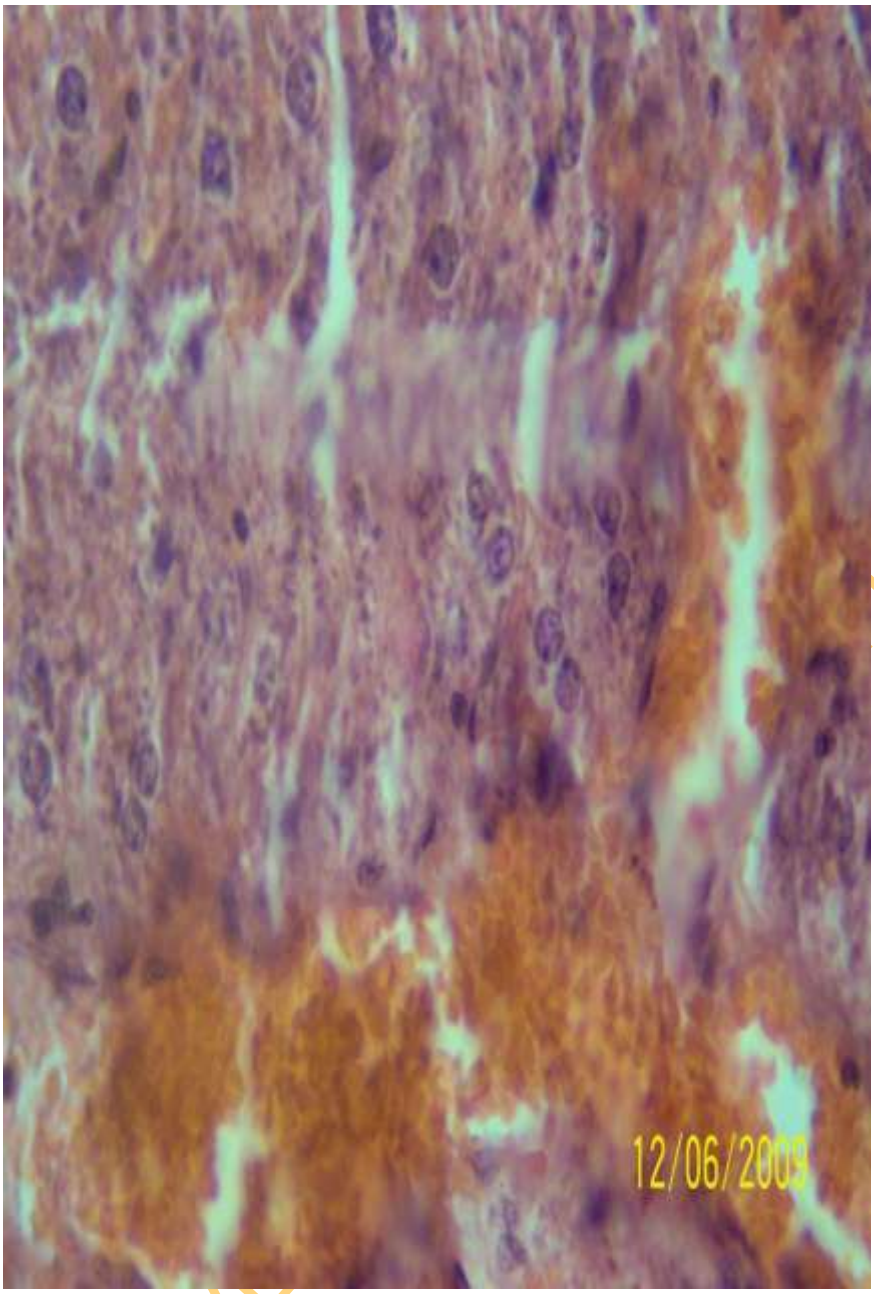

Figure4.11. Liver(H & E ×400) ofthe rat (control)administered orally 0.2ml of normal saline daily for 45days showing no visible lesion .

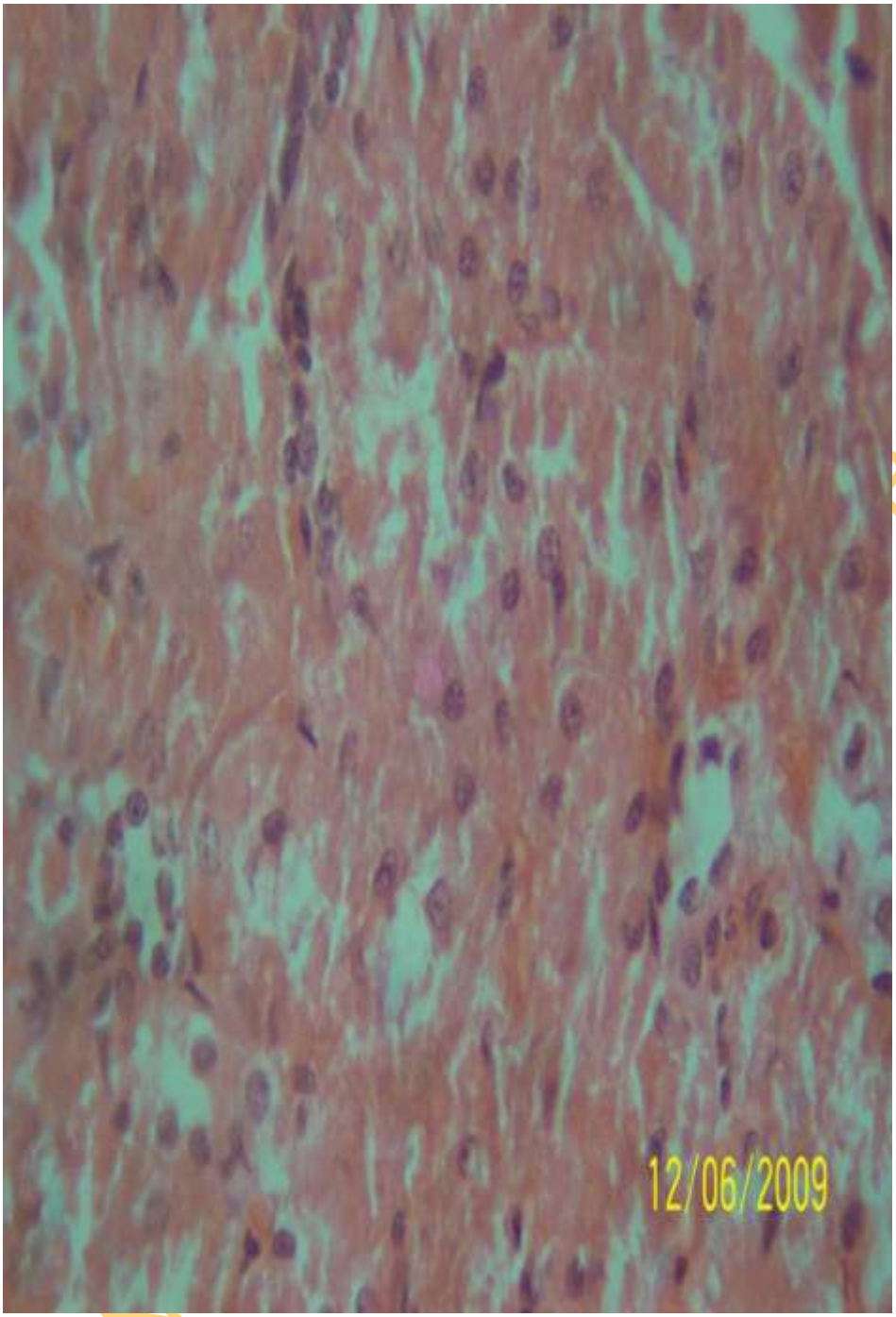

Figure4.12. Kidney(H & E ×400) of the (control)ratdosed orally 0.2ml of normal saline daily for 45days showing no visible lesion.

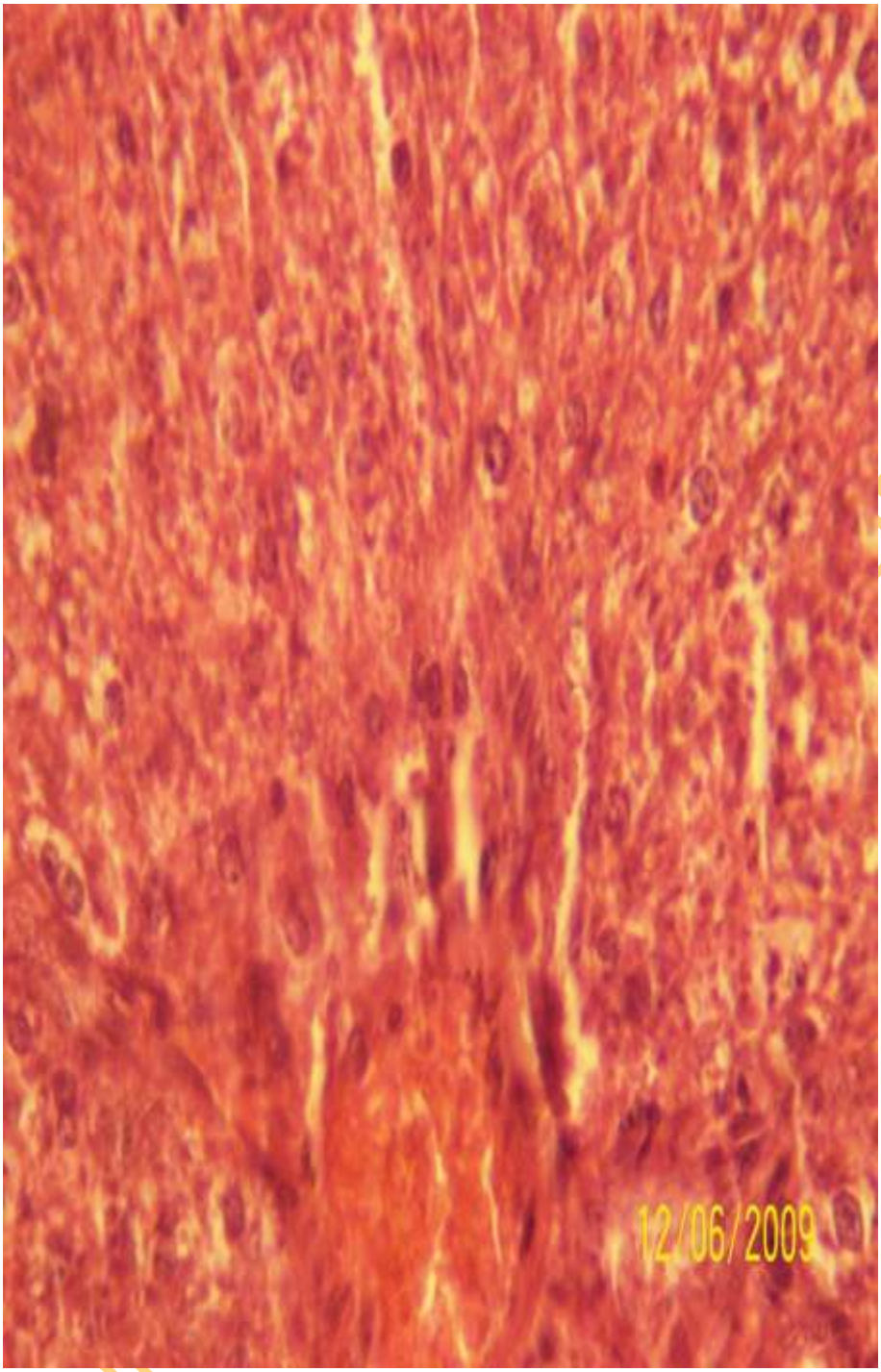

Figure4.13. Liver (H & E × 400) of the rat dosedorally 4mg 3TC/kg body weight daily for 45days showing no visible lesion.

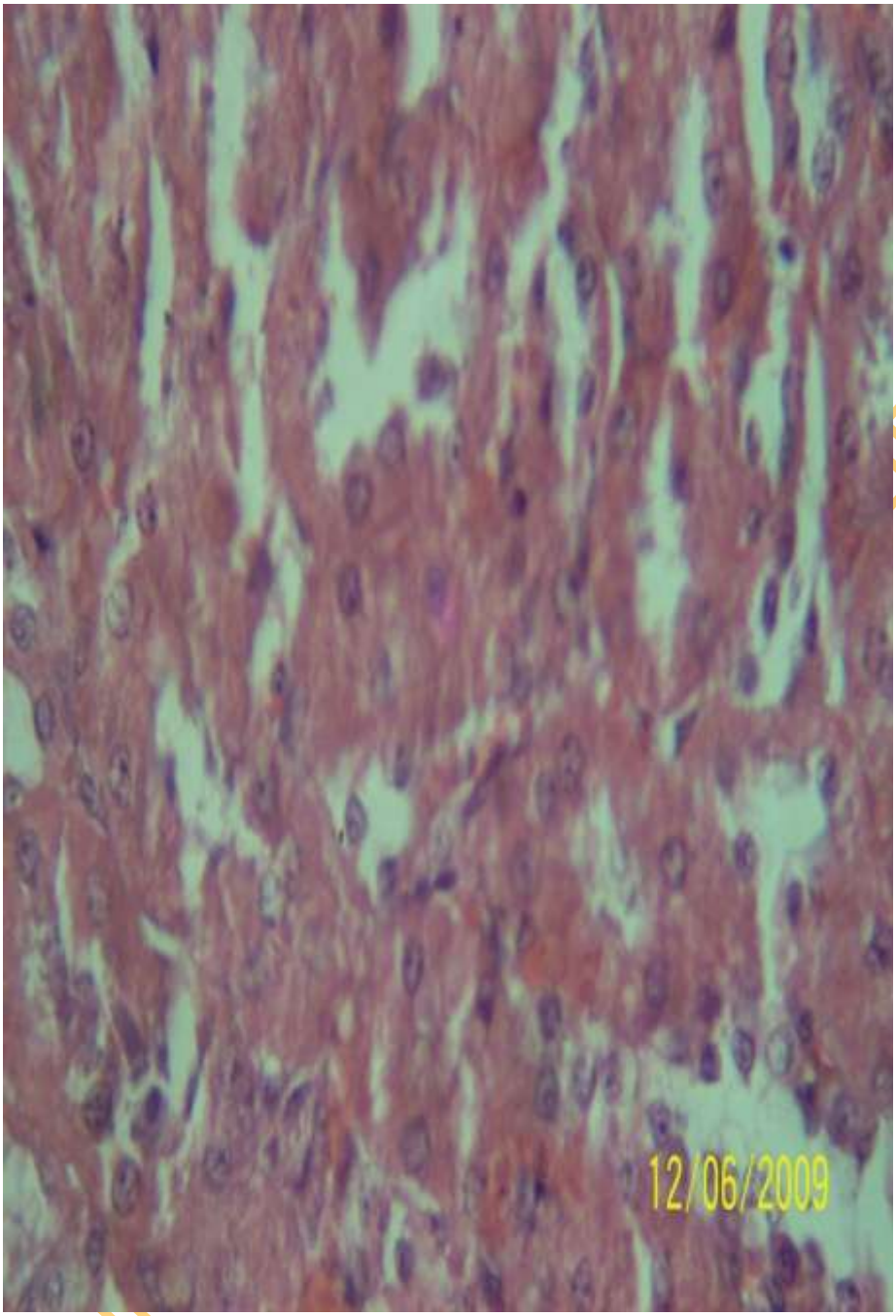

Figure4.14. Kidney (H & E ×400)of the ratdosedorally 4mg 3TC/kg body weightdaily for 45days showing no visible lesion.

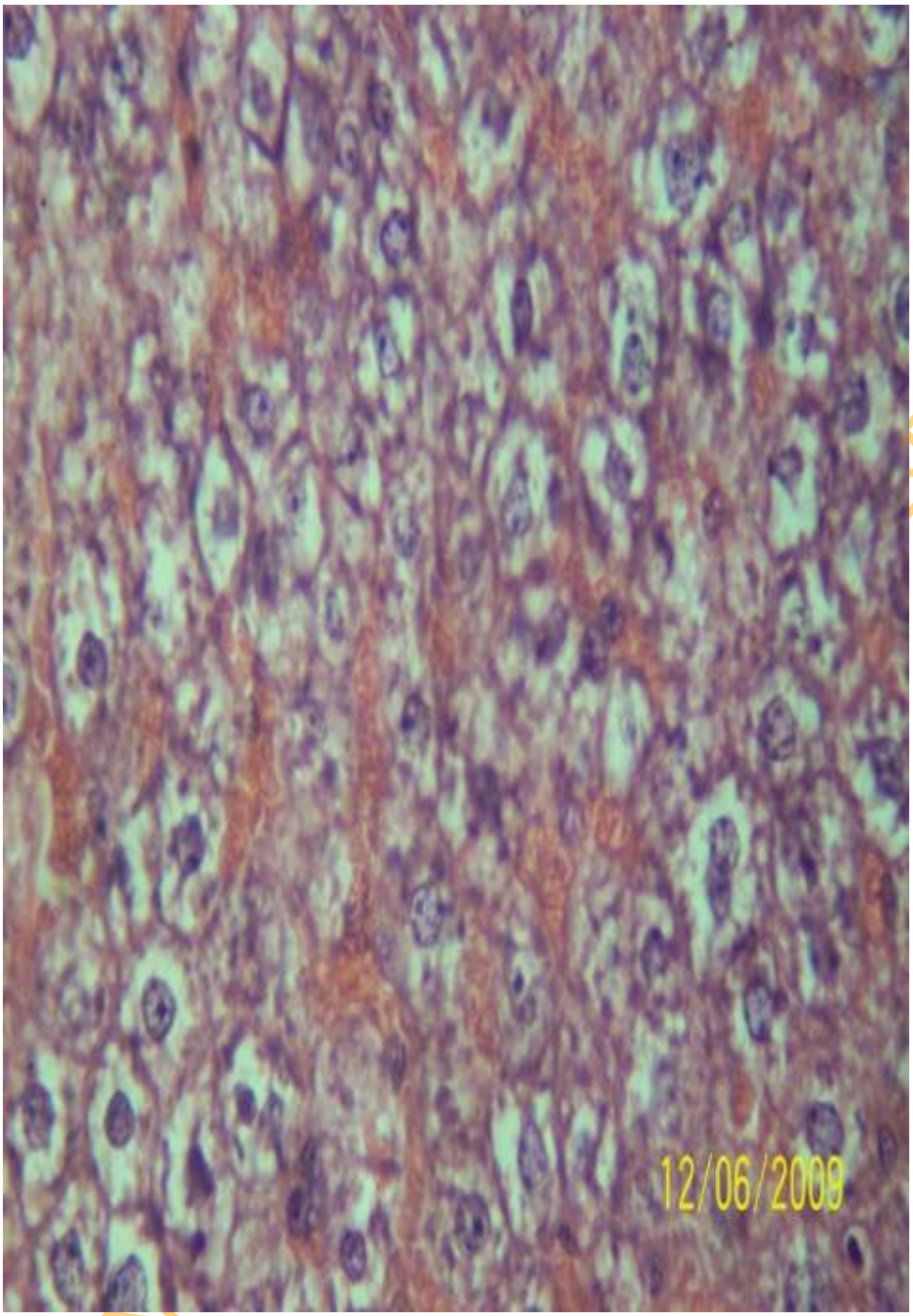

Figure4.15. Liver (H & E ×400) of the rat dosedorally 20mg 3TC/kg body weight dailyfor 45 days showing marked sinusoidal and portal congestion with diffuse hydropic degeneration of hepatocytes.

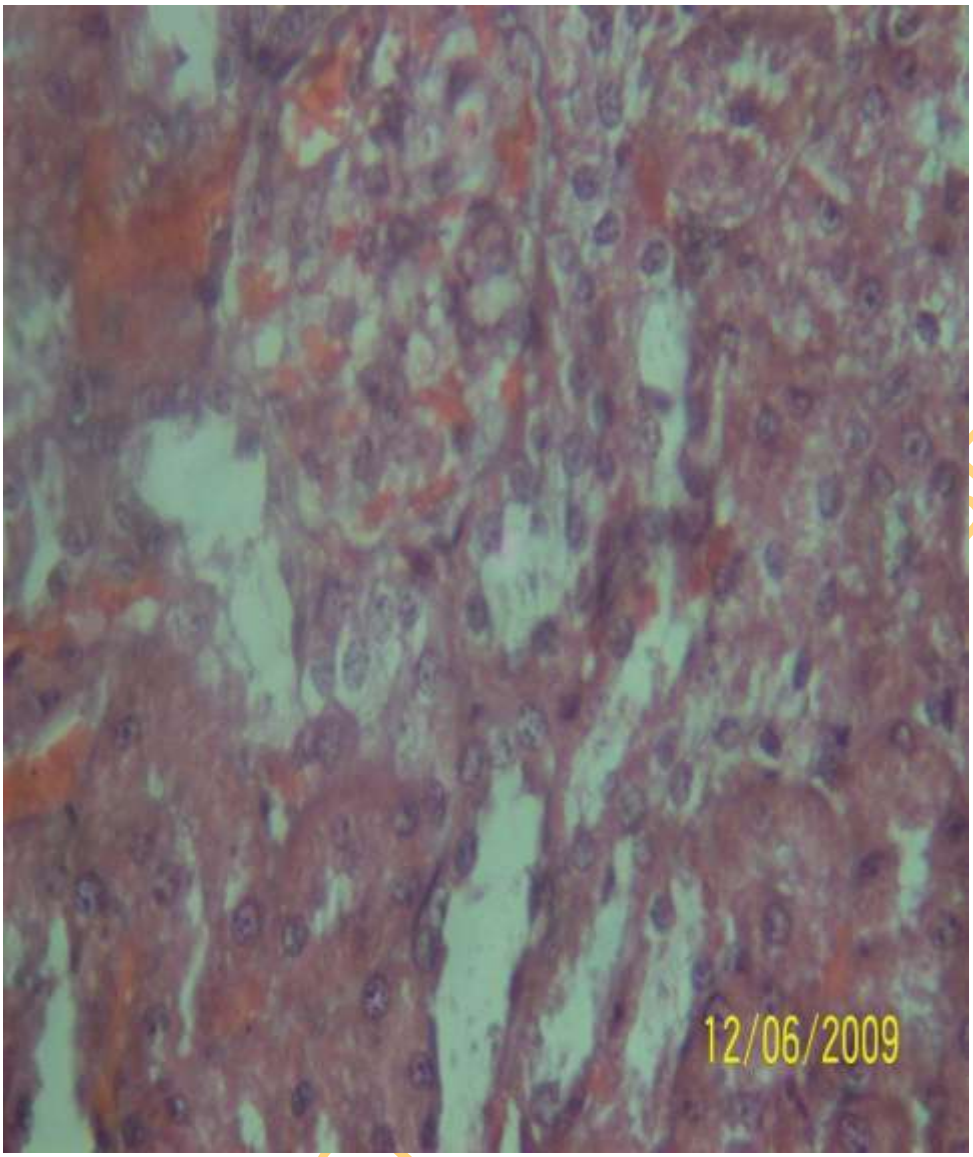

Figure4.16. Kidney (H & E ×400) of the rat following daily oral dose of 20mg 3TC/kgbody weight for 45daysshowingno visible lesion.

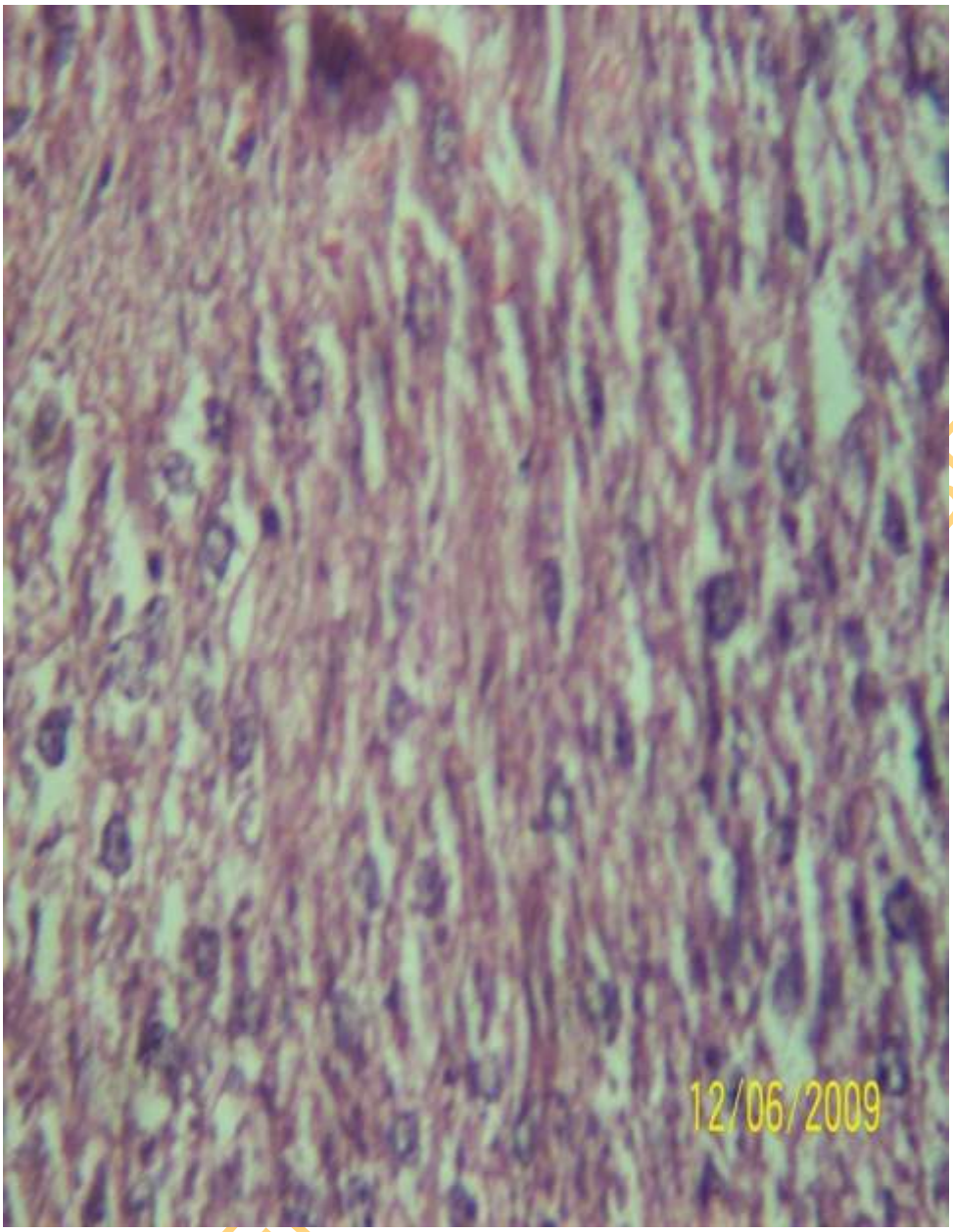

Figure4.17. Liver (H & E ×400) of the rat following daily oral dose of 100mg 3TC/kgbody weight for 45 days showing severe diffuse hydropic degeneration of hepatocytes.

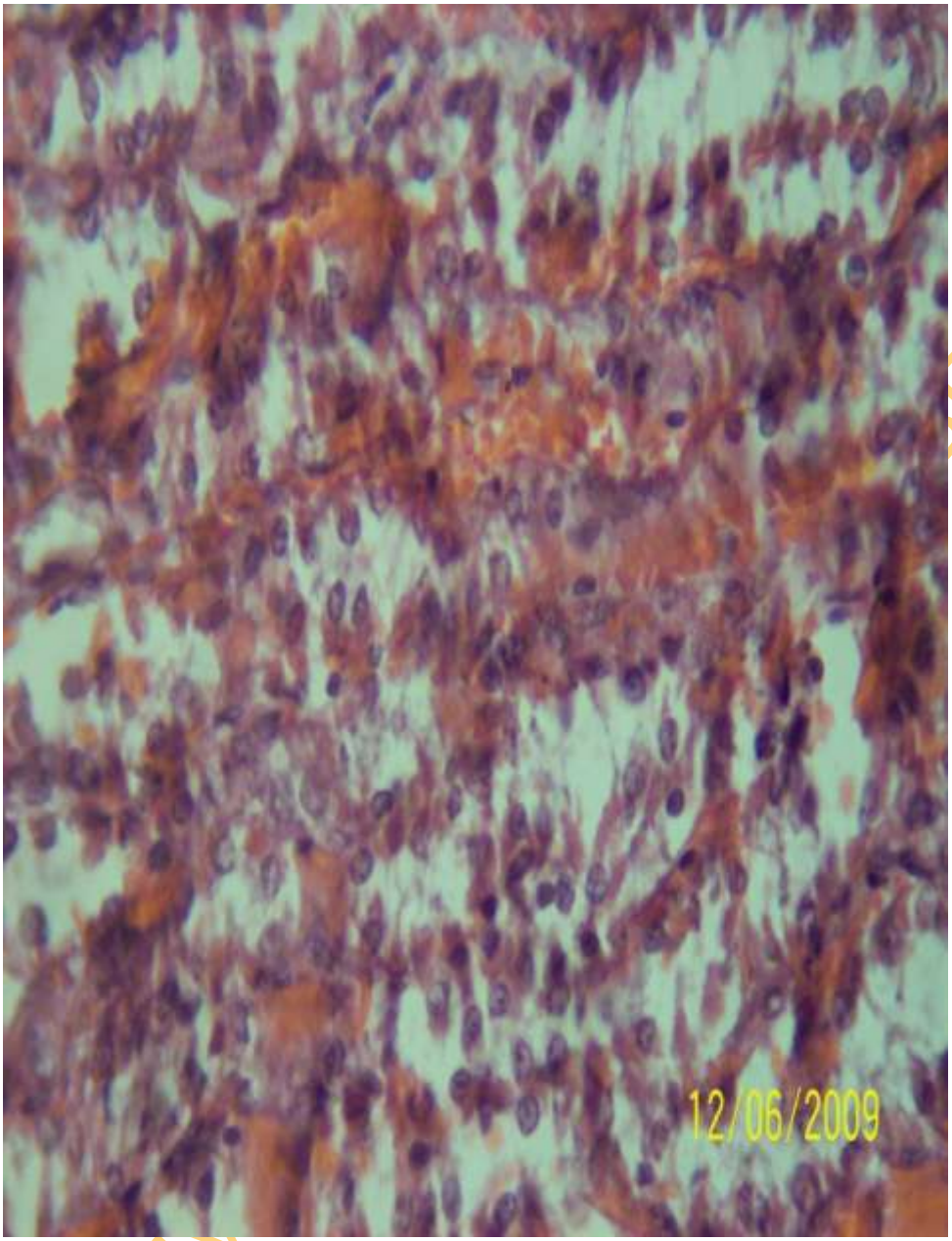

Figure4.18. Kidney (H & E ×400)of the rat dosed100mg 3TC/kg body weight dailyfor 45 days showing renal cortical congestion.

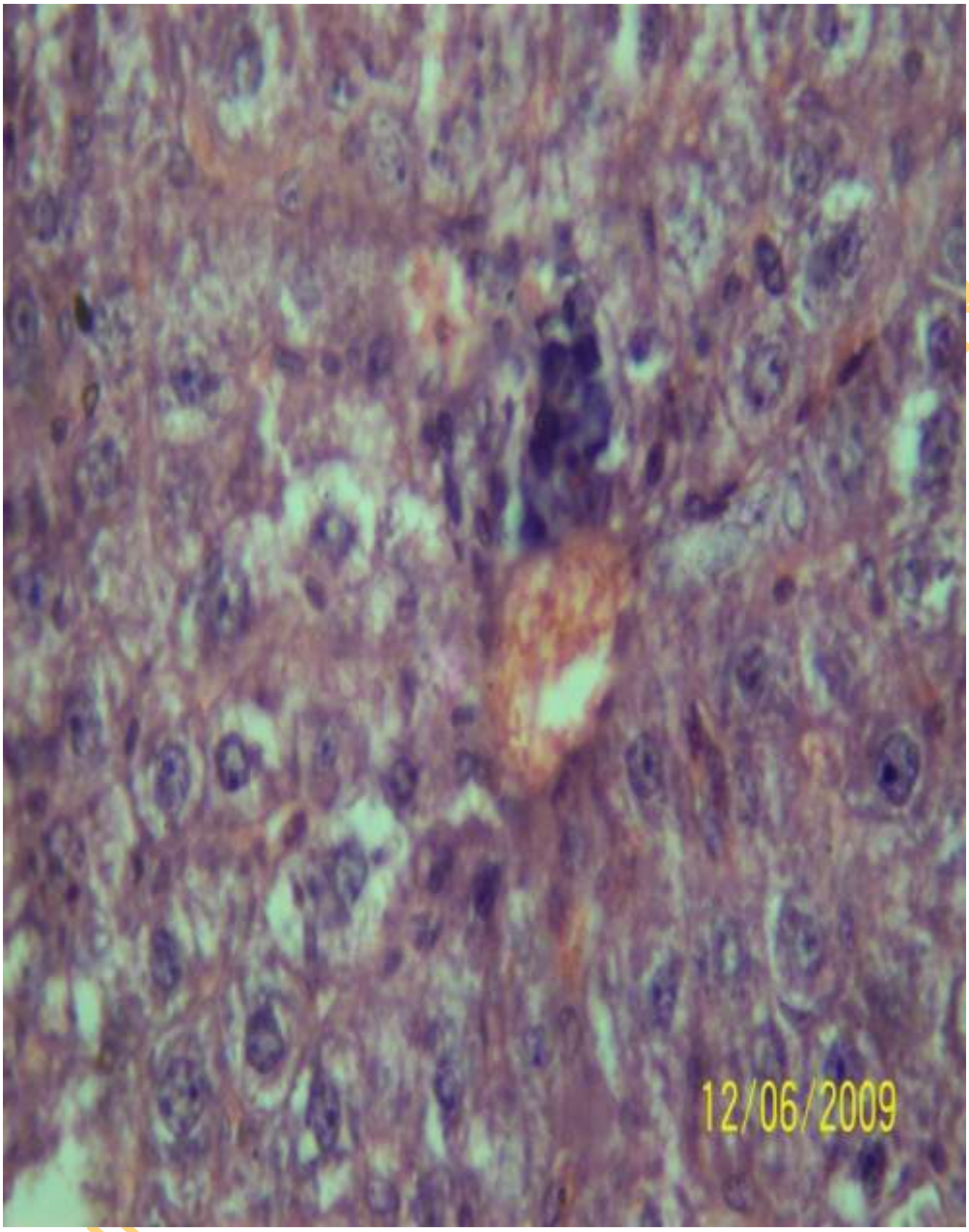

Figure4.19. Liver (H & E ×400) of the rat dosed500mg 3TC/kg body weight daily for 45days showing marked sinusoidal and portal congestion with severe hydropic degeneration of hepatocytes. There is also cellular infiltration (periportal) by macrophages.

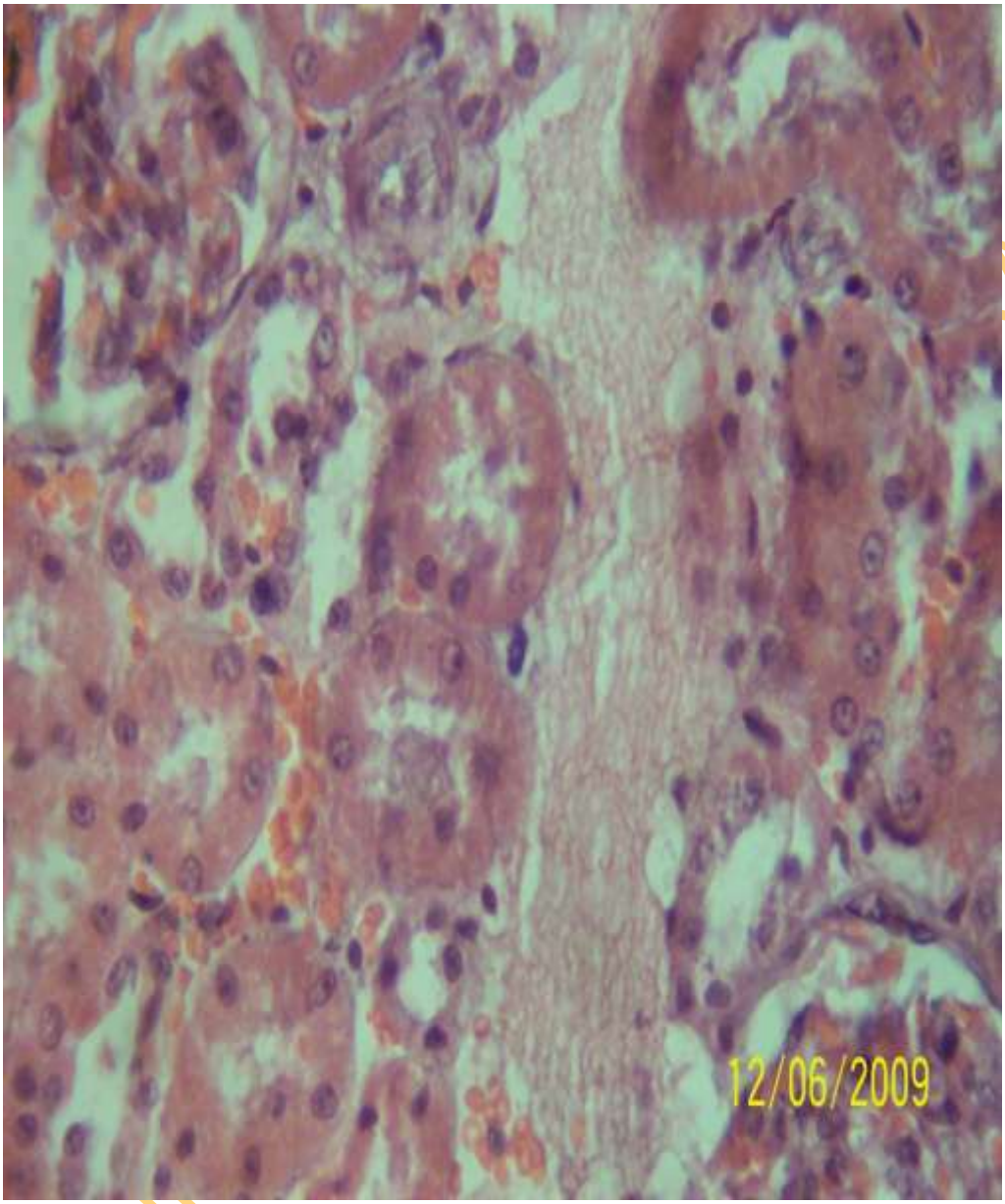

Figure4.20. Kidney(H&E x 400) of the rat dosed 500mg 3TC/kg body weight dailyfor 45days showing periglomerular congestion, fewprotein casts in the tubular lumen and pink staining fibrillar material in the renal interstitium.

CHAPTER FIVE

DISCUSSION

The fertile avian embryo is highly sensitive and susceptible to the toxic effectsof xenobiotics. In addition, the presence of phases 1 and 2 drug-metabolizing enzymes in the chicken embryo (Rifkind *et al*., 1994; Nakai *et al*., 1992) has made egg embryonation/egg titration technique one of choice in toxicity studies.The work of Spielmann (2002) appears to show that a positive correlation exists between egg titration technique and animal experiment in toxicity studies.The technique´s potential for generating drug metabolites was recently exploited (Dong *et al.*,2003). Egg titration study reduces pressure on the number of animals that would have been required for toxicity studies, a concept of "refinement, reduction and replacement"ofRussel and Burch (1959) now being integrated in the quest for alternative to the classical LD_{50}determination. These assertions may have justified our use of fertile embryo in the present study.

The $RD_{50}$of 427mg3TC/kg calculated from our *in vitro* study in the chicken embryo was rounded off to 500mg/kgfor the animal experiments. The rounding off to the whole number was embarked upon in order to comply with the geometric progression requirement of the moving average method of Weil (1952), which we have found convenient to use because the method is devoid of serious mathematical manipulations yet compares favourably with similar methods of determining LD_{50}. Also, the 500mg/kg adopted in this study is a part of the regimen of the Fixed Dose Procedure (Stallard and Whitehead, 2004), being considered in toxicologic studiesas an alternative to the classical procedure of LD_{50}determination.

Theobservedreductionin body weight - gain among the animals on repeated dosingrelative to the control animals or to those animals on single dosingcould be thought of as an adverse effect of the drug (Pijl and Meinder , 1996). Reduced adipose tissue has been the foremost cause of low body weight (Das and Vasudevan, 2005). Body-weight is determined by complex mechanisms regulating energy balance. A number of neurotransmitter systems acting in several hypothalamic nuclei are pivotal to the regulation of body - fat stores. Most drugs that are capable of changing body weight are known to interfere with these neurotransmitter systems. Lipoartrophy , loss of body fat which is related to reduced adipose tissue culminating in body - weight loss has been described as a clinical syndrome presenting among patients on NRTI drug treatment (Brinkman *et al.* , 1999). Lipoartrophy, an aspect of lipodystrophy (Carr, 1998) thought to be related to

mitochondrial dysfunction,has been proposed for NRTI toxicity (Lewis *et al.*, 2001) thoughit has not been scientifically proved for 3TC.

Urine analyses were carried out as tests of renal integrity during exposure to the drug. The expression of the urinary parameters on the basis of urinary creatinine concentration as reported in this investigation has been described as a valid technique of assessing tubular function (Vanderlagt *et al.* , 1992; Maruhn *etal.* , 1977) probably in view of diurnal /circadian changes in urine concentrates (Houser, 1986; Raab, 1972). Increased urinary magnesium (Mg) is pathological as well as a marker of nephrotoxicity. The thick ascending limb of Henle´s loop is the major site of Mg reabsorption. Increased urinary Mg may indicate leakage and therefore lesion at this site. The observed increase in urinary Mg/creatinine might indicate Mg leakage and therefore possible drug – induced lesion at the re-absorption site (Weichert – Jacobson *et al.*, 1999; Anand and Bashley, 1993). Urine trehalase, a lysosomal enzyme like N – acetyl - β-glucosaminidase (NAG) as well used as a marker of nephrotoxicity. Trehalase is located in the brush border membrane of renal proximal tubules (Berger, 1970) and is a recommended biomarker of nephropathy (WHO, 1991). Increased urinary trehalase activity is thought to indicate morphological damage at tubular brush borders (Niwa *et al.*, 1993; Nakano *et al.*, 1987). The observed positive correlation between urinary trehalase activity and protein/creatinine ratio ($p < 0.05$ and $p < 0.01$; $r = + 0.95$ at 100mg/kg and 500mg/kg respectively) suggested the presence of a nephrotic syndrome (Chavan *et al.*,2005), probably a transient tubular damage (Caliskan *et al.*,1996).

The increase in tissue - weight relative to the body - weight observed in the kidney and the livercould be related to lipodystrophy arising from abnormal mitochondrial metabolism. During mitochondrial dysfunction, oxidation of fatty acids is generally known to be impaired.

Changes in serum enzyme activities have for long been used in both clinical and scientific laboratories as markers of pathological changes in varioustissues where the enzymes are predominantly located. ALT and AST are present in high concentrations in the hepatocytes (Ringer *et al.*, 1979). The enzymes catalyse the reversible syntheses of alanine and aspartic acid. ALT, the more hepatospecific enzyme, is predominantly cytosolic. AST exhibits high activity in the cytoplasm, mitochondria and microsomes of liver, kidney and brain. These enzymes upon structural damage to the tissues are released into circulation (Sallie *et al.*, 1991; Benjamin, 1978),

hence theobserved respective steady increasesin their blood level activities among rats on repeated dosingin contrast to those on single dosingcould mean liver damage. The results of the analyses on the sera of animals on single dosingseemed to show recovery of the animals from the boost of the drug doses (John *et al.*, 2008).Serum quinine oxidase is a specific marker of toxic hepatitis (Ragno and Balde,1962; Balazs *et al.*, 1961; Roman and Dulmanis , 1959). The observed increase in the enzyme activity may havealso suggested drug – induced liver damage.The loss of serum total protein among the test rats compared with the control could be a reflection of negative protein turn – over which normally occurs under a pathological condition in the liver (Abatan *et al.*,1996).Gamma glutamyltransferase (GGT) functions in the catalysis of amino acids transfer across plasma membranes of renal tubules , bile ductule cells and also in the endoplasmic reticulum of hepatocytes

Amino acid + GSH —— $_{GGT}\rightarrow$ *γ- glutamylamino acid + cysteinylglycine* .

The increased serum level of this enzyme is consequence of its release from the liver cells during hepatobiliary diseases. The observed elevation of serum GGT activity after drug administration therefore suggested toxic hepatitis (Ruppin *et al.*, 1982).

Changes in the tissue GGT specific activity is also informative. For example, renal GGT activity has been used as an indicator of pathological changes in the proximal tubule (Pergande *et al.*,1994),the enzyme being found in the brush border of the proximal tubule. However, liver/kidney ratio of GGT activity has been demonstrated to be specie - specific. While the enzyme activity is higher in the liver cells than in the renal tubular cells in humans (Tsuchida *et al.*,1979), the reverse is the case for rats (Hinchman and Ballatori, 1990). GGT is GSH – dependent, being the only enzyme capable of excising gamma glutamate from GSH as illustrated in the equation above , and also in the generation of non-toxic mercapturate excretory product. Reduced glutathione (GSH) contributes significantly to the intracellular antioxidant defensive system as it is a powerful consumer of superoxide, singlet oxygen and hydroxyl radicals (Miesel and Zuber , 1993) . Together these consumable anions are called reactive oxygen species (ROS). Generation of ROS is a regularcellular occurrence during aerobiosis. In the mitochondria, ROS are generated via univalent reduction mechanism during electron transport. They become cytotoxic when they interact with biomolecules such as DNA, proteins and membrane lipids leading to certain dysfunction by the molecules. The breakdown of GSH – dependent

antioxidant system increases the intracellular flux of oxygen free radicals (Miesel *et al.*, 1995) creating an oxidative stress and initiating apoptosis.

Glutathione S – transferase (GST) like GGT is a GSH – dependent enzyme, present in large amounts in the liver and also in the renal cortex of the rat. The enzyme catalyses the conjugation of electrophilic xenobiotics with GSH making the xenobiotics water – soluble and excretable in water. The observed elevations of the mean specific activities of hepatic GGT and of both hepatic and renal GST among animals on repeated dosingwere sustained by the supply of GSH, probably an indication of adaptive response to oxidative challengeput up by the drug in the rat liver and kidney (Mohora *et al.,2002*).

Depressed GGT specific activityobserved in the renal tissues could be explained either as the result of GSH depletion or reduced intracellular GSH synthesis, both conditions are a marker of oxidative stress (Yu,1994), which has been implicated in some drug-induced nephropathy (Matsushima *et al.*,1998). The depressed GGT activity might not be drug – induced as the event was not statistically significant.

The GGT and GST responses in the liver were both significantly provoked by 20mg 3TC/kg whereas in the kidneyGST specific activity was significantly provoked by 100mg 3TC/kg. The dose differential in the GST responsesin the two tissues suggested drug – induced tissue sensitivity andconsequently marking the liver as being more affected by the drug than the kidney. Similarly , the change in GGT specific activity might mean absence of oxidative stress in the kidney at all the dose levels since GGT is more of renal than hepatic in rats (Hinchman and Ballatori , 1990) .

Lipid peroxidation (LPO), the oxidative breakdown of polyunsaturated fatty acids is a widely accepted mechanism for cellular injury and death. Malondialdehyde (MDA) and 4 – hydroxynonenal (HNE) are products of lipid peroxidation which have been found genotoxic through interaction with DNA leading to a final disruption of DNA base pairing. There is an inverse relationship between lipid peroxidation(exemplified by increased MDA concentration) and GSH levels (Maddaiah , 1990).The observed increase in MDA concentration in the kidney and liver tissues of the rats on repeated dosingsuggested lipid peroxidation which may lead to functional changesin these tissues (Reiter , 1995). The increased MDA concentration and the loss

of activity of GSH-dependent enzymes (GGT and GST), represented an inverse relationship between lipid peroxidation and GSH levels(Maddaiah,1990), an ingredient of oxidative stress in the tissues, though not statistically significant.

Superoxide dismutase (SOD),a cuproprotein widely distributed in mammalian tissues,is an antioxidant enzyme concerned with dismutation of superoxide anions ($O_2^{\cdot-}$) to H_2O_2 and O_2.The accompanied increase in SOD specific activities in the kidney and the liver could mean an adaptive responseto oxidative challenge by the drug.

In the brain, the observed increase in MDA concentration and loss of SOD specific activity suggested onset of oxidative stress (Yao *et al*., 1999 ; Mahdi *et al.* ,1996 ;Reddy *et al.*,1992) . However, the lack of statistical significance of the change in MDA concentration and SOD specific activity in the rat brain at all the dose levelsconsidered in this experiment ruled out drug – induced oxidative attack.Drug metabolism in the brain is determined by a number of factors including the well known blood – brain and blood – cerebrospinal fluid (choroid plexus and arachnoid membrane) barriers which together control drug entry into the brain vascular system and in particular, deficient oxidative defense mechanism , presence of high key ingredients of lipid peroxidation such as iron (Gupta e*t al.* , 2003), abundance of peroxidizable fatty acids , and high oxygen consumption (Skaper *et al.* , 1990)may make the brain vulnerable to oxidative attack. The interplay of these factors is expected to assume/play a definitive role in 3TC metabolism bythe brain.Isotopic study had shown that the 3TC volume of distribution in the brain was low (Wu *et al.,1998),* its concentration in the cerebrospinal fluid (CSF) had also been reported to be low (Mueller *et al.,1998*).The reason being that the concentration of NRTI family of anti – HIV drugs to which 3TC belongs, in the central nervous system is affected by the presence of efflux and influx transporter proteins at the brain barriers (Gibbs *et al.,2003a;*Gibbs and Thomas, 2002;Blaney *et al.,1995*). The effective control of drug entry into the brain could explain the diminished metabolic activity of the drug (3TC) in the brain as exemplified by the results from the various biochemical parameterswe investigated.

Rat brain mitochondria are known to abound in glutamate dehydrogenase (GDH). The enzyme is also present in the liver and kidney albeit at lower levels. Brain GDH is believed to contribute to the synthesis of the metabolic and neurotransmitter pools of glutamate. Rat brain GDH is especiallysensitiveto exogenous chemicals (Yoon *et al.*, 2002). The enzyme is able to bind many

compounds of diverse structures possibly playing a role in binding and consequently concentrating drugs in the mitochondria (Fahien and Shemisa, 1970). Although the specific activity of the brain GDH was elevated among the rats on repeated dosing, the increase was not statistically significant. The increased GDH activitymight have indicated absence of neurodegeneration, GDH activity is known to decrease during neurodegeneration (Cho, 2009).No satisfactory explanation could be offered in the present circumstancefor the observed loss in the rats brain weight *vis á vis* results from the biochemical parameters.Be that as it may, loss in weight generally spells a pathological condition, therefore assay of specific and more sensitive enzymes is desirable to give a definitive position.

The increase in tissue weight relative to the body weight observed in the kidney and the livercould be related to lipodystrophy arising from abnormal mitochondrial metabolism. During mitochondrial dysfunction, oxidation of fatty acids is known to be impaired.

CHAPTER SIX

6.0 SUMMARY AND CONCLUSIONS

The results of the *in vitro* and *in vivo* experiments might have suggested hepatotoxic and nephrotoxic potentials of lamivudine at doses ≥100mg/kg body weight when administered orally and repeatedly for ≥45 days in the female albino rats.

According to this study, drug-induced oxidative stress appeared to be the mechanism of toxicity operating in the liver and probably in the kidney, it is however an unlikely event in the brain at the dose levels considered.

Although the drug has been found to be a poor substrate for polymerase gamma ruling out mitochondrial toxicity, but at the drug high concentrations, the active site of the enzyme – polymerase gamma, the enzyme responsible for mitochondria synthesis,couldhowever become availableto the drug and thereby eliciting biochemical responses that approximate mitochondrial toxicity consequent of polymerase gamma inhibition. It is possible that other mechanism(s) than mitochondrial DNA synthesis inhibition was/were operating. Our data however did not suggestthe presence of drug – induced toxicity via oxidative stress or neurodegenerative lesion in the brain.This may require further study in view of the reduced brain weight relative to the body weight that was observed in this study.

According to the British Toxicological Society rating (Stallard and Whitehead,2004),500mg 3TC/kg taken for 45 days is 'harmful' to the female albino rats.

6.1 CONTRIBUTION TO KNOWLEDGE.

The recommended daily dose of 4mg/kg for Man is not toxic to the female rats when administered orally for a period as long as 45 days. Higher doses of the drug produce toxic lesions in the female rat kidney and liver.Drug-induced oxidative stress appears to be the

mechanism of toxicity in these organs. It appears to us the drug is not as toxic to the rat brainas in the liver and kidney at very high drug doses when administered orally for as long as 45 days.

6.2 FUTURE DIRECTION

Pharmacokinetic studies of interaction of lamivudine with certain drugs including cotrimoxazole, hydroxyurea and methotrexate when co – administered, have shown that lamivudine blood levels increase by as much as 44%. The toxicological implications, if any, of this on patients undergoing these treatments and the underlying mechanism(s), are not well understood. It is desirable to unravel this research quest for save and effective health – care delivery.

CHAPTER SEVEN

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